REMARKS/ARGUMENTS

Claims 10-14, 17, 19, 20, and 33-78 are active in this application. Support for the amendments to Claims 62, 66, 70, 64, 68, 72 and Claims 74-78 is found on page 3 of this application. In addition, the claims presented have been renumbered according to the Examiner's statement on page 2 of the Official Action. No new matter is added.

Applicants thank the Examiner for indicating that Claims 10-14, 17, 19, 20 and 33-61 are allowed. In light of the following remarks and the attached executed Declarations reconsideration of the rejection of Claims 63-73 is requested.

Applicants also thank the Examiner for the courtesy of discussing this case with Applicants' undersigned representative on March 9, 2004. During this discussion the Examiner suggested amending the scope of the method claims and consistent with that discussion, the claims have been amended to recite inflammatory arthritis, which is described and enabled by the application.

Before getting into the substance of the rejection, Applicants request that the Examiner reconsider the finality of the outstanding Office Action. As a basis for issuing a final Office Action following the request for continued examination, the Examiner states "all claims are drawn to the same invention claimed in the application prior to entry of the submission under 37 C.F.R. § 1.114 and could have been finally rejected on the grounds of art of record in the next Office Action if they had been entered in the application prior to entry under 37 C.F.R. § 1.114." (Page 16 of the Official Action). However, the request for continued examination was filed because the Examiner stated in the Advisory Action of August 19, 2003 (paper No. 29) that the proposed amendments would not be entered because "they raise new issues that would require further consideration and/or search" and particularly "amended and newly added claims raise new issues with respect to the scope of

the claims since the claims are now limited to specific compounds administered *in vivo* to treat an inflammatory condition arthritis or contacted antagonizing or agonizing a C5a receptor. However, such *in vivo* administration to treat the above situations were not dealt with previously, and as such would require further consideration and search." Therefore, the finality of the Office Action seems to run contrary to the opinion of the Examiner in the Advisory Action. In other words, the RCE was filed because the Examiner would not enter the amendments and then issued a first action final rejection. Not only is this unfair to the Applicants but it is deemed to be improper and therefore it is respectfully requested that the finality of the rejection be withdrawn.

Turning to the rejection of Claims 62-73 under 35 U.S.C. § 112, first paragraph, this rejection is respectfully traversed for the following reasons.

As stated previously, Claims 62-63 are enabled because the Applicants have provided *in vivo* data using well-accepted models for assessing anti-inflammatory and anti-arthritis effects. The data presented in the application are shown in Examples 7 and 8 on pages 45-46. In particular, Example 7 demonstrates the inhibition of C5a induced neutropenia *in vivo* using one of the inventive compounds. In addition on page 46, the cyclic antagonists in Table 5 were determined to be active anti-inflammatory agents in suppressing the onset of either carrageenan-induced paw oedema or adjuvant-induced polyarthritis.

On page 46, the Applicants have also shown in the carrageenan paw oedema assay "that even weak C5a antagonists significantly inhibits development of the oedema after 180 and 270 minutes." Moreover, in a recent publication, which is of record, data are presented which demonstrate the efficacy of compound No. 12 in an antigen-induced arthritis model in the rat.

Notwithstanding these data, the Examiner has maintained the enablement rejection alleging that the specification "does not reasonably provide enablement for a method of

treating an inflammatory condition or a method of treating arthritis by administering the above compounds thereof as claimed in Claims 62-73." (Page 3 of the Official Action). Furthermore, the Examiner has stated that "the quantity of experimentation necessary states the phrases "treating inflammatory condition" and "treating arthritis" are not justified by the limited exemplary disclosure of suppressing the onset of either carrageenan-induced paw oedema or adjuvant-induced polyarthritis as disclosed in Figures 8-10 in Examples 7 and 8 because the above phrases encompass treating any kind of inflammation (unspecified inflammation caused by unspecified agent) as well as any kind of arthritis (undefined arthritis) using pharmaceutical formulations..." (page 7 of the Official Action). Applicants respectfully disagree.

First, as noted above, the claims have been amended to recite "inflammatory arthritis" and Claims 74-78 further define the inflammatory arthritis as "rheumatoid arthritis." Second, as discussed above, the data presented in the originally filed specification clearly enables these methods. However, as further support for the claimed methods, Applicants submit herewith two Declarations from Dr. Steven Taylor and Dr. Vivian Santer attesting to the enablement of the method claims in the current application.

Dr. Taylor has an extensive history and knowledge in inflammation research as shown by his statement attached to the Declaration as Appendix A and his *curriculum vitae* attached as Exhibit SMT-1.

Applicants direct the Examiner's attention to paragraph 5 on page 2 of Dr. Taylor's Declaration where he states:

> I consider that in fact the model systems described in the specification are very well accepted as being generally predicative of anti-inflammatory efficacy, not only in the treatment of rheumatoid arthritis but in other inflammatory conditions. Moreover, I consider that efficacy and the treatment of rheumatoid arthritis is regarded as being reasonably predicative of efficacy and other inflammatory arthritides.

Furthermore, the Examiner's attention is drawn to paragraph 7 of Dr. Taylor's Declaration (found on page 2) in which he describes the performance of additional assays both *in vitro* and in the rat carrageenan paw oedema model and rat adjuvant arthritis model described in the specification demonstrating that compounds of the invention have anti-inflammatory and anti-arthritic activity. The results are presented in a published manuscript and are attached to Dr. Taylor's Declaration as Exhibit SMT-2.

With respect to the predicative value of the rat paw oedema assay the Examiner's attention is directed to paragraph 11 of Dr. Taylor's Declaration (found on page 3) wherein he states

> I emphasize that the *in vitro* assays described in the specification in the rat paw oedema model are general models useful for testing anti-inflammatory activity, and are not restricted to assessment of efficacy in the treatment of arthritis. They are therefore useful as preliminary tests for efficacy in *any* inflammatory condition. (Emphasis in original)

This statement by Dr. Taylor confirms Applicants' position that the models provided in the specification support the enablement of the claimed compounds for treating inflammatory arthritis. Further discussion of the predictive value of the models used in the specification is discussed by Dr. Taylor in paragraphs 12-13 (pages 3-4) in which he describes why the antigen induced arthritis models are physiologically relevant based on similarities between pathologies associated in the model with the actual disease conditions. Dr. Taylor also points to the literature which demonstrates that the antigen induced arthritis model is a well-established experimental model of arthritis as well as other anti-inflammatory and immune based therapies (see paragraph 14 on page 4 of the Declaration). He refers to these articles as Exhibits SMT-3, SMT-4 and SMT-5.

On page 5, paragraph 16, Dr. Taylor states

> I therefore believe that a person of ordinary skill in the art would, once in possession of the present specification, have a

> reasonable expectation that the compounds of the invention would be useful and the treatment of numerous inflammatory conditions, especially several inflammatory arthritis, and most notably rheumatoid arthritides.

Dr. Taylor also provides data which are attached to the Declaration as Exhibit SMT-6. The data demonstrated that:

> antigen induced arthritis as being an established model of RA that involves stimulation of T-lymphocyte reactivity against the immunizing antigen. This model is induced by the immunization of animals with a protein antigen (methylated bovine serum albumin, ovalbumin or fibrin) and an adjuvant, followed by the intra-articular injection of the same antigen. This results in an immune-complex mediated inflammatory response, characterized by chronic synovitis, which is localized through the injected joint. (See paragraph 19 on page 5 of Dr. Taylor's Declaration).

Further evidence that the rat carrageenan-induced acute model of inflammation is useful for testing and assessing anti-inflammatory activity is provided as Exhibit SMT-7 of Dr. Taylor's Declaration. Exhibit SMT-7 is a copy of the U.S. Food and Drug Administrations Center for Biologics Evaluation Research and Guidance for Industry on "Clinical Development Programs for Drugs, Devices and Biological Products for the Treatment of Rheumatoid Arthritis (RA)." In particular, the Examiner's attention is drawn to paragraph 20 (page 6) of the Declaration wherein Dr. Taylor summarizes these guidelines in relation to the disclosure of the present specification:

> the rat carrageenan-induced acute model of inflammation is stated to be useful in assessing anti-inflammatory activity, and it is also stated that most of the animal models which involve inflammation in the paw may be used for measuring antiphlogistic (i.e., anti-inflammatory) action of a drug. This is the carrageenan-induced footpad inflammation model which is described in the present specification.

As further evidence that this model is predicative of rheumatoid arthritis and anti-inflammatories, in general, Dr. Taylor notes in paragraph 21 (page 6) that there are hundreds

of publications using the carrageenan-induced arthritis model for testing anti-inflammatory compounds.

In view of this extensive discussion of the knowledge in the field and the data of record in this application, Dr. Taylor, in paragraph 24 (page 7) concludes:

> I therefore consider that a person of ordinary skill in the art would, once in possession of the present specification, have a reasonable expectation that the compounds of the invention would be useful in the treatment of numerous inflammatory conditions, especially several inflammatory arthritis, and most notably rheumatoid arthritis. Moreover, such a person would readily be able to test efficacy of the compounds of the invention for this purpose in well-established experimental models, without the need to exercise any further inventive effort. Accordingly, I consider that the specification provides an enabling disclosure of a method of treating inflammatory arthritis, comprising the step of administering an effective amount of a compound of the invention to a mammal in need thereof.

Turning to the Declaration of Dr. Santer, it is noted that Dr. Santer also has an extensive history and knowledge in the field of connective tissue and arthritis research which is supported by the copy of her *curriculum vitae* attached to her Declaration as Exhibit VBS-1. In particular, the Examiner's attention is drawn to paragraphs 11, 12, and 13 (pages 3-4) of Dr. Santer's statements in which she confirms that

> the carrageenan-induced footpad oedema is a widely used standard assay for assessing the anti-inflammatory activity of candidate drugs for this indication, and that adjuvant-induced arthritis is also widely used for this purpose. . . For example, the rat footpad model was used for the initial demonstration of the anti-inflammatory activity of indomethacinn piroxicam, to well-known non-steroidal anti-inflammatory drugs (NSAIDs) which are very commonly used in the treatment of rheumatoid arthritis and other inflammatory arthritises, such as psoriatic arthritis and ankylosing spondylitis.

Information sheets for the NSAIDs are attached to Dr. Santer's Declaration as Exhibit VBS-4.

Based on Dr. Santer's extensive background and knowledge of the field, she concludes

> I therefore consider that long before the priority date, the experimental models described in the specification in respect of the present application were widely known in the art, and regarded as reasonably predictive of results in humans.

In view of (1) the above discussion, (2) the description in the specification, (3) the data of record in this application, (4) the Declaration of Dr. Taylor and supporting Exhibits, and (5) the Declaration of Dr. Santer and supporting Exhibits, the methods claimed in Claims 62-73 are unquestionably enabled by the specification as originally filed. Therefore, Applicants respectfully request withdrawal of this ground of rejection.

Upon withdrawal of this rejection, Applicants also request that this application be allowed. Early notice of such allowance is requested.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.
Norman F. Oblon

Daniel J. Pereira, Ph.D.
Registration No. 45,518

Customer Number
22850

Tel: (703) 413-3000
Fax: (703) 413 -2220
(OSMMN 08/03)

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No.
09/446,109 by The
University of Queensland

STATUTORY DECLARATION UNDER RULE 132

I, Vivien Bedford Santer of 21 High Road, Camberwell, in the State of Victoria 3124, Commonwealth of Australia, do solemnly and sincerely declare as follows:

1. I am a registered patent attorney and a member of the firm of Griffith Hack of 509 St Kilda Road, Melbourne, in the State of Victoria 3004, Commonwealth of Australia (hereinafter referred to as my firm), which is acting on behalf of the assignee of the present application, and I am the patent attorney primarily responsible for prosecution of this application in Australia and in other countries. I am responsible for instructing the applicant's United States attorneys, Messrs. Oblon Spivak McClelland Maier & Neustadt PC.

2. I have also worked in the field of medical research for many years, including several years of research in the field of connective tissue and arthritis research. A copy of my *curriculum vitae* is now shown to me, and is annexed hereto as Exhibit VBS-1.

3. I have read and understood the Office Action dated 18 December 2003 issued in respect of this application.

4. In this Office Action, the Examiner has raised an objection under Section 112 first paragraph that the specification does not provide an enabling disclosure in respect of a method of treating an inflammatory condition, an arthritic condition, or an inflammatory arthritis, comprising the step of administering an effective amount of a compound of the invention to a mammal in need thereof.

5. From January 1974 to November 1976 I was a Senior Tutor in the Department of Biochemistry at Monash University in Melbourne, Australia and worked in the Connective Tissue Group of the Department. This group had for a number of years used carrageenin-induced arthritis in rabbits as a model system for rheumatoid arthritis. Members of this research group were investigating various aspects of pathological changes in cartilage morphology and metabolism, and the group had published many papers on results obtained using this model.

6. From 1980 to 1981 I was a post-doctoral fellow in the Joint Diseases Laboratory at the Shrine's Hospital for Crippled Children in Montreal, Canada, *inter alia* carrying out studies on protoglycans in human articular cartilage.

7. During these two periods I was familiar with the scientific literature in the field, and maintained an awareness of new developments. In particular, I was aware of the animal model systems which were being used in the study of rheumatoid arthritis and other inflammatory arthritides. For example, I was aware of the use of the seaweed polysaccharide carrageenin to induce a variety of inflammatory responses. These included granulomas induced by subcutaneous injection of carrageenin; footpad oedema induced by sub-plantar injection of carrageenin into the footpad of rats; and adjuvant-induced arthritis in rats or mice. All of these models used small rodents, and consequently the types of study which could be carried out were limited by the size of the joint.

8. For this reason, the laboratory at Monash University had adopted a model of arthritis induced by intra-articular injection of carrageenin into rabbits. This model had already been used at Monash University for some years before I joined the laboratory, and I myself used this model. I was co-author on two publications in which this model

was used, and copies of abstracts of these publications are annexed hereto as Exhibit VBS-2.

9. This model has only partially been superceded by antigen-induced arthritis, which was initially described in 1962. I was involved in introducing this model to the laboratory at Monash University in about 1975-1976, and I was a co-author of a publication reporting studies using the antigen-induced arthritis model in rabbits. A copy of the abstract of this publication is annexed hereto as Exhibit VBS-3.

10. Through my reading of the scientific literature and through my subsequent work as a patent attorney in respect of patent applications relating to methods and therapeutic agents for the treatment of rheumatoid arthritis and other inflammatory arthritides, I was aware well before the priority date of the present application that collagen-induced arthritis is regarded as the most closely related form of antigen-induced arthritis to rheumatoid arthritis. However, I was aware both from the literature and from the work of my colleagues at Monash University that collagen is a protein which is very difficult to prepare in highly-purified form. Consequently I was aware that other antigens, such as ovalbumin or albumin are much cheaper and easier to obtain, and that such antigens are more commonly used in the art, at least in preliminary studies.

11. I was also aware that carrageenin-induced footpad oedema is a widely used standard assay for assessing the anti-inflammatory activity of candidate drugs for this indication, and that adjuvant-induced arthritis is also widely used for this purpose. I was aware that these assays are widely regarded in the art as being predictive of efficacy in the treatment of inflammatory conditions, including inflammatory arthritides.

12. For example, the rat footpad model was used for the initial demonstration of the anti-inflammatory activity of indomethacin and piroxicam, two well-known non-steroidal anti-inflammatory drugs (NSAIDs) which are very commonly used in the treatment of rheumatoid arthritis and other inflammatory arthritides, such as psoriatic arthritis and ankylosing spondylitis. Proprietary information sheets referring to this which were found on the World Wide Web are annexed hereto as Exhibit VBS-4.

13. I therefore consider that long before the priority date the experimental models described in the specification in respect of the present application were widely known in the art, and regarded as reasonably predictive of results in humans.

And I make this solemn Declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by the Act for the making of false statements in Statutory Declarations, conscientiously believing the statements contained in this Declaration to be true in every particular.

DECLARED at Melbourne this 13th day of May 2004

.....
Vivien Bedford Santer

Before me: .....

SALLY ANN SHRIMPTON
3rd Floor, 509 St. Kilda Rd, Melbourne 3004
A current practitioner within the meaning
of the Legal Practice Act 1996.

A person empowered to witness
Statutory Declarations under the
laws of the State of Victoria,
Commonwealth of Australia

EXHIBIT 1

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT VBS-1

This is Exhibit VBS-1 referred to in the Statutory Declaration by Vivien Bedford Santer

dated 13th May 2004

Before me:

Sally Shrimpton

SALLY ANN SHRIMPTON
3rd Floor, 509 St. Kilda Rd, Melbourne 3004
A current practitioner within the meaning of the Legal Practice Act 1996.

A person empowered to witness Statutory Declarations under the laws of the Victoria, Commonwealth of Australia

CURRICULUM VITAE - DR VIVIEN SANTER

Date of Birth: February 6th, 1949

University Education:

B.Sc. University of Melbourne, 1967-1969.

First year:	Pure Mathematics I	Honours IIB
	Chemistry IA	Honours IIA
	Physics IA	Honours III
	Biology	Honours I
	N.B. - Chemistry IA and Physics IA were Honours level subjects.	
Second year:	Chemistry IIB (second in class)	Honours I
	Physiology and Biochemistry (This subject included a Histology unit)	Honours III
	General Microbiology	Honours I
Third Year:	Biochemistry II	Honours IIA
	Virology	Honours I
	Bacteriology	Honours IIA
	Australian Society for Microbiology Prize for Virology	

B.Sc. (Hons) University of Melbourne 1970

A research project was carried out in the Electron Microscopy Laboratory at the Walter and Eliza Hall Institute of Medical Research under the supervision of Dr T.E. Mandel. The Honours thesis was entitled "Light and electron microscope studies on antigen-binding *in vitro* in the immune response".

Received Honours IIA.

Ph.D. - University of Melbourne 1974.

Research work was carried out in the same laboratory under the supervision of Professor G.J.V. Nossal (now Sir Gustav Nossal) and Dr T.E. Mandel. The thesis was entitled "Ultra-structural and functional studies on lymphocyte membranes: surface immunoglobulin receptors and the surface coat".

Positions Held:

(1) Senior Tutor, Department of Biochemistry, Monash University, Melbourne, Australia.

January 1974 - November 1976

Subject of Research

1. Light and electron microscope studies on carrageenin-induced arthritis and antigen-induced arthritis in the rabbit. These systems are used as models for human rheumatoid arthritis.

2. Radioautographic studies on cartilage metabolism in model systems for arthritis.

The position entailed extensive tutoring and laboratory class demonstrating to second year Science students, plus some lecturing.

(2) Research Associate, Department of Anatomy, McGill University, Montreal, Canada.

January 1977 - In Dr P.K. Lala's laboratory

Subject of Research

1. Spontaneous fusion between Ehrlich ascites tumour cells and host cells *in vivo*, using surface H-2 antigens as a marker detected by sandwich radioimmunolabelling and radioautography.

2. Studies on lymphocyte populations in mice bearing transplanted or spontaneous tumours, with reference to their subsets as determined by labelling with anti-Ig, anti-Thy 1.2, and anti-Ly antibodies.

3. Studies on the function of lymphocytes from mice with transplanted and spontaneous tumours in natural cytotoxicity assays *in vitro* and *in vivo*.

Dr Santer was in charge of the laboratory from June 1977 to July 1978 during Dr Lala's absence on sabbatical leave; this involved supervision of two M.Sc. students and two technicians

From September 1978 to October 1979 she was jointly responsible for supervision of a B.Sc. (Hons.) student.

(3) Post-doctoral Fellow, Joint Diseases Laboratory, Shriners' Hospital for Crippled Children, Montreal, Canada. In collaboration with Dr P.J. Roughley, carrying out studies on the proteoglycans of connective tissues.

Subject of Research

1. Studies on changes in the physical and chemical properties of proteoglycans from normal and degenerate cartilage of the human tibial plateau.

2. Studies on the physical and chemical properties of proteoglycans from normal human articular cartilage and their changes with age.

3. Studies on the oligosaccharide moiety of human articular cartilage proteoglycan.

4. Studies on a latent proteoglycan-degrading enzyme in medium from human fibroblasts and synovial cells cultured in the presence of mononuclear cell factor (in collaboration with Dr Elaine Golds).

(4) Research Fellow, Departments of Physiology and Biochemistry, Monash University, 1982 (Part-time).

In collaboration with Dr C.J. Handley, Ms A. Fosang and Professors D.A. Lowther and G.D. Thorburn, carrying out morphological studies on changes occurring in the uterine cervix of the sheep during gestation and parturition.

(5) Research Fellow, Dean's Department, Faculty of Medicine, Monash University 1983 (Part-time).

In collaboration with the Dean of Medicine, Professor G.C. Schofield, performing electron microscopic studies on acid secretion in the stomach, and on the effect of Epidermal Growth Factor on gastric parietal and chief cells.

(6) Joined Griffith Hack (then called Clement Hack & Co.), Patent and Trade Mark Attorneys, Melbourne in June 1984 as a Technical Assistant.

Completed the examinations for registration as a Patent Attorney in 1986, with Distinctions in Patents and Designs, and Credit in Trade Marks.

Registered as a Patent Attorney, December 1986.

Fellow of the Institute of Patent Attorneys of Australia, February 1987.

Became a partner of the firm in July 1990.

Responsible for preparation, filing and prosecution of Australian and foreign patent applications in all fields relating to biotechnology, particularly biochemistry,

microbiology, and immunology, as well as general pharmacology and pharmaceutical chemistry; patent oppositions, searches, general advice, opinions and litigation support in these fields.

Teaching Experience:

- Part-time demonstrator in General Microbiology 1970-1971 (University of Melbourne).
- Tutor and demonstrator in Biochemistry 203 1974-1976 (Monash University).
- Lecturer in Biochemistry 205 1975-1976 (Monash University).
- Supervision of B.Sc (Hons.) students 1974-1975 (Monash University); 1978 (McGill University).
- Supervision of M.Sc. students 1977-1978 (McGill University).

Other Activities

Dr Santer was invited to be the first Chairman of the Patents and Licensing Working Party of the Australian Biotechnology Association, and held that position from the inception of the Working Party in 1987 until 1997. The Working Party liases between the academic and industrial research communities and the patent and legal professions, and has made submissions to the Biotechnology Consultative Group of the Australian Department of Industry, Technology and Commerce and to the Minister on various matters. In1997 the Working Party was replaced by the Intellectual Property Group, of which Dr Santer is a member. Dr Santer contributes a regular column to the bi-monthly Journal of the Association, and is a member of the organising committee of the Association's Victorian branch. Dr Santer was also a member of the Victorian Organising Committee of the Intellectual and Industrial Property Society from 1987 to 1989.

Dr Santer has given lectures in management of intellectual property in the CSIRO training course for Divisional business managers and senior scientific staff, and frequently gives presentations on patenting at scientific and other conference, as well as contributing papers on patenting and intellectual property to journals in the biotechnology field.

In 1995, Dr Santer accepted a Ministerial appointment to the Board of Patent Attorneys Professional Standards, and was the primary examiner for the Patents subject for registration as a patent attorney from 1995 to 1997.

Since 1996 Dr Santer has been a non-executive director of Rothschild's Australasian Bioscience Investment Trust, now independent of Rothschild and called GBS Venture Partners Ltd.

In March 2003 Dr Santer accepted an invitation to join the Advisory Committee on genetics, intellectual property rights and human health care, assisting the Australian Law Reform Commission with a reference from the Federal Government on "Intellectual Property Rights Over Genetic Material and Genetic and Related Technologies"

Membership of Professional Societies:

- Australian Society for Biochemistry and Molecular Biology

- Australian Biotechnology Association
- Intellectual Property Society of Australia and New Zealand
- Licensing Executives' Society
-International Federation of Industrial Property Attorneys
-Asian Patent Attorneys' Association

PUBLICATIONS

1. Marchalonis, J.J., R.E. Cone and V. Santer.
"Enzymic Iodination. A Probe for Accessible Surface Proteins of Normal and Neoplastic Lymphocytes"
Biochem. J. 1971 124 921.

2. Santer, V., A.D. Bankhurst and G.J.V. Nossal.
"Ultrastructural Distribution of Surface Immunoglobulin Determinants on Mouse Lymphoid Cells"
Exptl. Cell Research 1972 72 377.

3. Santer, V., R.E. Cone and J.J. Marchalonis.
"The Glycoprotein Surface Coat on Different Classes of Murine Lymphocytes"
Exptl. Cell Research 1973 79 404.

4. Santer, V.
"Ultrastructural Radioautographic Studies on Capping and Endocytosis by Mouse Spleen Lymphocytes"
Aust. J. Exp. Biol. Med. Sci. 1974 52 241.

5. Santer, V.
"The Surface Coat on Mouse Lymphocytes"
Proc. 8th International Congress on Electron Microscopy, Canberra, 1974 Vol. II p. 242.

6. Mandel, T.E. and V. Santer.
"Ultrastructural of the Lymphocyte Surface" In The Lymphocyte- Structure and Function"
Ed. J.J. Marchalonis (Marcel Dekker Inc., N.Y.) 1977 pp. 511-529.

7. Santer, V.
"The Cell Coat or Glycocalyx of Lymphocytes".
Ibid. pp. 541-564.

8. Carmichael, D.J., G.C. Gillard, D.A. Lowther, C.J. Handley and V. Santer.
"Carrageenin-Induced arthritis. IV. Rate Changes in Cartilage Matrix Proteoglycan Synthesis"
Arth. Rheum. 1977 20 834.

9. Lowther, D.A., J.D. Sandy, V. Santer and H.L.G. Brown.
"Antigen-Induced Arthritis: Decreased Proteoglycan Content and Inhibition of Proteoglycan Synthesis in Articular Cartilage"
Arthr. Rheum. 1978 21 675.

10. Lala, P.K., S. Garnis, L. Kaizer, S. Jacobs and V. Santer
Characterization of Lymphocytes Invading Experimental Tumours. Proceedings of the International Conference on Germinal Centers in Immune Responses, Damp, West Germany, 1978.
Adv. Exp. Med. Biol. 1979 114 778-781.

11. Santer, V., J.H. Mastromarino and P.K. Lala.
"Characterization of Lymphocyte Subsets in Spontaneous Mouse Mammary Tumours and Host Lymphoid Organs"
Int. J. Cancer 1980 25 159-168.

12. Lala, P.K., V. Santer and K.S. Rahil
"Spontaneous Fusion Between Ehrlich Ascites Tumour Cells and Host Cells *In Vivo*: Kinetics of Hybridisation and Concurrent Changes in the Histocompatibility Profile of the Tumour after Propagation in Different Host Strains"
Eur. J. Cancer 1980 16 387-510.

13. Chatterjee-Hasrouni, S., V. Santer and P.K. Lala.
"Changes in the Maternal Lymphoid System During Syngeneic and Allogeneic Pregnancy in the Mouse"
Cell. Immunol. 1980 50 290-304.

14. Santer, V., J.H. Mastromarino and P.K. Lala.
"Characterization of Small Lymphocyte Subsets in Mice Bearing Spontaneous Mammary Tumours"
Fed. Proc. 1979 38 992.

15. Lala, P.K., V. Santer and J. Mastromarino.
"Characterization of Small Lymphocyte Subsets in Mice Bearing Spontaneous Mammary Tumours"
Annual Meeting of the Canadian Federation of Biological Societies, 1979.

16. Osmond, D.G., V. Santer and N. Saveriano.
"Radioautographic Studies of Lectin Binding by Small Lymphocytes in Mouse Bone Marrow, Thymus and Spleen"
Annual Meeting of the Canadian Federation of Biological Societies, 1979.

17. Saveriano, N., N. Drinnan, V. Santer and D.G. Osmond.
"Lectin-Binding Patterns of Small Lymphocytes in Bone Marrow, Thymus and Spleen: Demonstration of Lymphocyte Subsets by Quantitative Radioautogrpahy"
Eur. J. Immunol. 1981 11 870-876.

18. Santer, V., P.J. Roughley and R. White
"Proteoglycans from Normal and Degenerate Cartilage of the Human Tibial Plateau"
Arthr. Rheum. 1981 24 691-700.

19. Roughley, P.J., R. White and V. Santer
"Comparison of Proteoglycans Extracted from High and Low Weight- Bearing Human Articular Cartilage, with Particular Reference to Sialic Acid Content"
J. Biol. Chem. 1981 256 12690-12704

20. Roughley, R.J., D. McNicol, V. Santer and J. Buckwalter.
"The Presence of a Cartilage-Like Proteoglycan in the Adult Human Meniscus"
Biochem. J. 1981 197 77-83.

21. Santer, V., E. Golds, R.J. White and P.J. Roughley.
"Mononuclear Cell Factor (MCF) Stimulates Release of a Proteoglycan- Degrading Enzyme form Human Skin Fibroblasts and Synovial Cells"
Arthr. Rheum. 1981 24 S118.

22. Osmond, D.G., N. Saveriano, M. Drinnan, V. Santer, M.D. Rahal, J.J.T. Owen and A.M. Rijnbeek.
"Lectin Binding by Bone Marrow Lymphocytes: Pre-B Cells have a Surface Receptor for Peanut Agglutinin"
Developments in Immunology 1981 15 103-110.

23. Santer, V., R.J. White and P.J. Roughley.
"O-Linked Oligosaccharides of Human Articular Cartilage Proteoglcyan"
Biochem. Biophys. Acta 1982 716 277-282.

24. Santer, V., A. Sriratana and D.A. Lowther.
"Carrageenin-Induced Arthritis: V. A Morphological Study of the Development of Inflammation in Acute Arthritis"
Seminars in Arthritis and Rheumatism. 1983 18 162-168.

25. Fosang, A.J., C.J. Handley, V. Santer, D.A. Lowther, C.M.L. Harper and G.D. Thorburn.
"Pregnancy-Related Changes in the Connective Tissue of the Ovine Cervix"
Biol Reproduction 1984 30 1223-1235.

26. Golds, E., V. Santer, J. Killackey and P.J. Roughley.
"Mononuclear Cell Factors Stimulate the Concomitant Secretion of Distinct Latent Proteoglycan, Gelatin and Collagen Degrading Enzymes from Human Skin Fibroblasts and Synovial Cells"
J. Rheumatol 1983 10 861-871.

27. Santer, V.
"Patents and Patenting - A Primer for the Biotechnologist"
Aust. J. Biotechnol 1988 1 25-30.

28. Santer, V.
"Problems in Obtaining Patent Protection in Biotechnology"
Proceedings of the 8th Australian Biotechnology Conference, February 1989.

29. Santer, V.
"Problems in Obtaining Patent Protection for Biotechnology "
Conference of the Institute of Patent Attorneys of Australia, March 1989.

30. Santer, V.
"Intellectual Property Protection for Plants - Patents and Plant Variety Rights"
Workshop on Intellectual Property Protection for Plants, July 1989.

31. Santer, V.
"Patents for Higher Animals and Plants"
Proceedings of the 9th Australian Biotechnology Conference, September 1990.

32. Santer, V.
"Patents for Higher Animals and Plants"
Conference on Ethics and Intellectual Property, Melbourne, October 1990.

33. Santer, V.
"Patents in Biotechnology"
Search 1993 24 77-81.

34. Santer, V.
Patents and the Human Genome Project: Implications of the Venter Application
Proceedings of the 11th Australian Biotechnology Conference, September 1993

35. Cox, G., S.S.H. Wong, V.B. Santer,
"Patentability of Biotechnology Inventions in Countries of the Pacific Rim"
Australasian Biotechnology, 1994 4 (5) 274-277

36. Santer, V.
"Does the Patent System Affect Research?"
Proceedings of the Pacific Rim Biotechnology Conference, February 1995.

37. Santer, V
"Strategies for Maximising Patent Life'
Second Australia/ Japan Symposium on Drug Design and Development, September 1996.

38. Santer, V.
"Intellectual Property Rights For Transgenic Crops"
Workshop on Commercialisation of Transgenic Crops, March 1997

39. Santer, V.
"Where the Wild Things Are: The Tension Between Patents and Traditional Knowledge"
Proceedings of the 14th Australasian Biotechnology Conference, April 1998.

40. Santer,V.
"Developments in Australian Patent Law"
Biotechnology Industry Organisation Conference, New York, June 1998.

41. Santer, V.
"Protecting Inventions in Combinatorial Chemistry"
Third Australia-Japan Symposium on Drug Design and Development, Tokushima, September 1998.

42. Santer,V.
"Recent changes in patent law: how do they impact on researchers and companies"

Conference on Advances in Diagnostic Technologies, Melbourne, May 1999

43. Santer,V.
"Recent changes in patent law: how do they impact on researchers and companies"
Commercialisng Health Innovations Forum, Melbourne, October 1999.

44. Santer,V.
"Intellectual Property and Patent Issues"
in "Australian Biotechnology Report 1999" (Ernst & Young, 1999)

45. Santer,V.
"Patentability of genomics, bioinformatics and research tools"
Biotechnology Industry Organisation Conference, Boston, March 2000.

46. Santer,V.
"American Booty: Crossing the Threshold from Discovery to Patentable Invention"
Fourth Australia/ Japan Symposium on Drug Design and Development, May 2000.

47. Santer,V.
"Bioprospecting, Traditional Rights and Patents"
15th Australasian Biotechnology Conference, July 2000.

48. Santer,V.
"Bioprospecting, Traditional Rights and Patents"
International Marine Biotechnology Conference, September 2000.

49. Santer,V.
"Patents for Research Tools: Problem or Promise?"
15th Australasian Biotechnology Conference, July 2001.

50. Santer,V.
"Cost–Effective Foreign Patenting"
Fifth Australia/ Japan Symposium on Drug Design and Development, May 2002.

51. Santer,V.
"Are genomics patents worthwhile?"
AusBiotech conference, August 2002

SUBMISSIONS ON BEHALF OF THE AUSTRALIAN BIOTECHNOLOGY ASSOCIATION

1. Senate Select Committee on Agricultural and Veterinary Chemicals in Australia, 1989.

2. Plant Variety Rights Office: re proposed revisions to the UPOV Convention, 21 July 1989.

3. Department of Foreign Affairs and Trade: re TRIPS Negotiations, 1990.

4. Expert Study of Legal Protection of Plants in Australia under Patent and Plant Variety Rights Legislation on behalf of the Patent Office and Plant Variety Rights Office, 24 August 1990.

5. House of Representatives Standing Committee on Industry, Science and Technology Inquiry into Genetically Modified Organisms (co-author), 16 November 1990.

6. U.S. Association of Biotechnology Companies Patent Law Working Group re US Patent Law Reform and re Durden Case - submitted to U.S. Advisory Commission on Patent Law Reform, 12 July 1991.

7. Proposed amendments to the Environment Protection Act and the Gene Technology Bill (April 2000).

8. Access to Australia's biological resources in Commonwealth areas (April 2000).

9. Intellectual Property and Competition Review Committee interim report (May 2000).

EXHIBIT 2

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT VBS-2

This is Exhibit VBS-2 referred to in the Statutory Declaration by Vivien Bedford Santer

dated 13th May, 2004

Before me:

Sally Shrimpton

SALLY ANN SHRIMPTON
3rd Floor, 509 St. Kilda Rd, Melbourne 3004
A current practitioner within the meaning of the Legal Practice Act 1996.

A person empowered to witness Statutory Declarations under the laws of the Victoria, Commonwealth of Australia

Entrez PubMed
Overview
Help | FAQ
Tutorial
New/Noteworthy
E-Utilities

PubMed Services
Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
LinkOut
Cubby

Related Resources
Order Documents
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

Privacy Policy

☐ 1: Semin Arthritis Rheum. 1983 Nov;13(2):160-8.

Related Articles, Links

Carrageenin-induced arthritis: V. A morphologic study of the development of inflammation in acute arthritis.

Santer V, Sriratana A, Lowther DA.

Following a single injection of the polysaccharide carrageenin into the rabbit knee joint, a rapid inflammatory process occurs in the joint space and synovial membrane, followed by changes in the articular cartilage. Initially there is an influx of cells, mainly PMNs, into the synovial fluid, accompanied by proliferation of the synovial lining cells and infiltration of the synovial membrane. The numbers of synovial fluid cells decline gradually after 24 hr. The reaction in the synovial membrane is greatest at day 7, and inflammation is still evident at day 21. Initially, the infiltrate consists mainly of PMNs, but by day 7 it is predominantly mononuclear, with small clusters of lymphocytes. The articular cartilage shows loss of metachromasia with toluidine blue at 3-14 days after injection, but stains normally after day 21. Electron microscopy shows damage to the chondrocytes at day 1 and 7, with complete destruction of cells in the surface layer. At day 7 cells in the deeper layers have lost the apparatus required for proteoglycan synthesis, but at day 21 the cells appear virtually normal. There was no evidence for a direct inhibitory effect of carrageenin on proteoglycan biosynthesis. Most labeled carrageenin was rapidly cleared from the joint space, but about 10% was retained in the synovial membrane and 0.6% in articular cartilage at 48 hr after injection. Since the increase and decline in PMN numbers respectively precede the cartilage damage and recovery, it is suggested that there may be a correlation between the clinical activity of arthritis and the number of PMNs in the synovial fluid.

PMID: 6673111 [PubMed - indexed for MEDLINE]

☐ 3: Arthritis Rheum. 1977 Apr;20(3):834-40. Related Articles, Links

Carrageenin-induced arthritis. IV. Rate changes in cartilage matrix proteoglycan synthesis.

Carmichael DJ, Gillard GC, Lowther DA, Handley CJ, Santer VB.

A localized inflammatory response was initiated by both single and repeated injections of carrageenin into femorotibial joints. Histologic changes were observed 24 hours after a single intraarticular injection, and an inhibition in the in vitro rate of proteoglycan synthesis was detected 72 hours after the injection. This inhibition was relieved in vitro by the addition of beta-D-xyloside, an exogenous initiator of glycosaminoglycan biosynthesis. Following repeated carrageenin injections, most cells appeared to be dead on histologic examination and no in vitro proteoglycan synthesis could be detected; nor could any stimulation be achieved by adding exyloside.

PMID: 856216 [PubMed - indexed for MEDLINE]

EXHIBIT 3

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT VBS-3

This is Exhibit VBS-3 referred to in the Statutory Declaration by Vivien Bedford Santer

dated 13th May, 2004

Before me:

Sally Shrimpton

SALLY ANN SHRIMPTON
3rd Floor, 509 St. Kilda Rd, Melbourne 3004
A current practitioner within the meaning
of the Legal Practice Act 1996.

A person empowered to witness Statutory
Declarations under the laws of the Victoria,
Commonwealth of Australia

☐ 2: Arthritis Rheum. 1978 Jul-Aug;21(6):675-80. Related Articles, Links

Antigen-induced arthritis. Decreased proteoglycan content and inhibition of proteoglycan synthesis in articular cartilage.

Lowther DA, Sandy JD, Santer VB, Brown HL.

An arthritis was induced in rabbits immunized with human serum albumin by injection of antigen into the hind knee joint. Histological changes in the synovial membrane and an increase in polymorphonuclear granulocytes in the synovial fluid indicated an inflammatory response similar to that described with fibrin as antigen (1). The arthritis was accompanied by no significant change in the collagen content, but a marked decrease in the proteoglycan content of the cartilage was noted. Cartilage from inflamed joints generally exhibited a decreased ability to synthesize proteoglycan in vitro.

PMID: 736998 [PubMed - indexed for MEDLINE]

EXHIBIT 4

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT VBS-4

This is Exhibit VBS-4 referred to in the Statutory Declaration by Vivien Bedford Santer

dated 13th May 2004

Before me:

Sally Shrimpton

SALLY ANN SHRIMPTON
3rd Floor, 509 St. Kilda Rd, Melbourne 3004
A current practitioner within the meaning
of the Legal Practice Act 1996.

A person empowered to witness Statutory
Declarations under the laws of the Victoria,
Commonwealth of Australia

INFORMATION FOR
HEALTH PROFESSIONALS

Data Sheet

RHEUMACIN

Indomethacin

Presentation

25 mg Capsules: white OP body, white OP cap, size 3. Contents a white powder.

50 mg Capsules: white OP body, white OP cap, size 2. Contents a white powder.

75 mg Capsules: clear colourless body, clear yellow cap, size 2. Contents small off-white spheres.

Uses

Actions

RHEUMACIN (indomethacin) is a highly effective nonsteroidal anti-inflammatory medicine with marked analgesic and antipyretic properties.

INDOMETHACIN is a potent inhibitor of prostaglandin synthesis *in vitro*. Concentrations are reached during therapy which have been demonstrated to have an effect *in vivo* as well.

INDOMETHACIN has been shown to be effective anti-inflammatory agent, appropriate for long-term use in rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis.

INDOMETHACIN affords relief of symptoms; it has not been shown to alter the progressive course of the underlying disease.

INDOMETHACIN has been found effective in relieving the pain, reducing the fever, swelling, redness, and tenderness of acute gouty arthritis.

The prostaglandin-inhibitory effect of INDOMETHACIN has been shown to be useful in the relief of pain and associated symptoms of primary dysmenorrhoea.

Anti-inflammatory Action:

The anti-inflammatory activity of INDOMETHACIN was first demonstrated in animals, measuring the ability of the compound to inhibit either granuloma formation or oedema induced by subplantar injection of carrageenin in rats. The latter appears to correlate well with antirheumatic activity in humans. Assays of relative potency indicated that INDOMETHACIN was more potent than acetylsalicylic acid, phenylbutazone or hydrocortisone; the potency ratios differed with the test employed.

The inhibition of carrageenin-induced oedema by INDOMETHACIN is specific the compound failed to inhibit oedema induced by a variety of agents other than cargeenin, nor did it reduce oedema if the medicine was administered after the oedema had been established.

As with other anti-inflammatory agents, the mechanism of action of INDOMETHACIN is unknown. INDOMETHACIN is fully active in the absence of the adrenals and the activity is readily demonstrable by direct application of the compound to the site of action. Unlike anti-inflammatory steroids, INDOMETHACIN in intact animals did not affect the size of the adrenals or the thymus, nor did it retard gain in body weight; these are sensitive indications of adrenal activation. The anti-inflammatory activity of combinations of

INDOMETHACIN and a steroid was that of either medicine alone in comparable doses.

Experiments have shown INDOMETHACIN to have a favourable effect upon adjuvant-induced polyarthritis in rats; it was more active than phenylbutazone or acetylsalicylic acid in suppressing the delayed manifestations of disseminated arthritis. This response is said to correlate well with clinical antiarthritic activity.

Antipyretic Activity:

The antipyretic activity of INDOMETHACIN has been demonstrated in rabbits and rats injected with bacterial pyrogen, and in the classical yeast-induced fever assay in rats.

A direct comparison of peak antipyretic activity in the yeast fever test showed INDOMETHACIN to be about nine times as potent as aminopyrine, 24 times as potent as phenylbutazone, and 43 times as potent as acetylsalicylic acid.

The antipyretic activity of INDOMETHACIN has been confirmed clinically by observations in patients with a variety of febrile conditions.

Analgesic Activity:

INDOMETHACIN is active in animal tests designed to assay analgesic activity of non-narcotic analgesics. Moderate doses raise the response threshold when pressure is applied to the yeast-inflamed foot of the rat, but do not affect responses to thermal stimuli, or to pressure on a non-inflamed foot. Qualitatively, INDOMETHACIN behaves like an analgesic of the anti-inflammatory/antipyretic type typified by the salicylates, and not of the narcotic type typified by morphine.

When single oral doses of INDOMETHACIN were assayed in the inflamed foot assay, the compound was found to be about 28 times as potent as acetylsalicylic acid and about 14 times as potent as phenylbutazone.

Pharmacokinetics

INDOMETHACIN is well absorbed after oral administration in all animals. In dogs, monkeys and rats, peak plasma levels after an oral dose occur within 0.5 to 2 hours.

The route of excretion is related to the species of animal and is independent of the route of administration or size of dose. Nearly all the compound or medicine-related metabolites could be recovered in urine and faeces. The rabbit eliminates INDOMETHACIN almost entirely in the urine, while the dog excretes nearly all the compound in the faeces. The rat, guinea pig, and monkey eliminate it by both routes.

In rabbits, rats, guinea pigs, and monkeys some INDOMETHACIN is metabolised by deacylation or demethylation and the metabolites are excreted as such or as the glucuronide conjugate.

Clinical Studies:

Prostaglandins sensitise afferent nerves and potentiate the action of bradykinin in inducing pain in animal models. Moreover, prostaglandins are known to be among the mediators of inflammation. Since INDOMETHACIN is an inhibitor of prostaglandin synthesis, the mode of action may be due to a decrease of prostaglandins in peripheral tissues.

In patients treated with INDOMETHACIN for rheumatoid arthritis and osteoarthritis, the anti-inflammatory action of INDOMETHACIN has been shown by reduction in joint swelling, reduction in pain, reduction in duration of morning stiffness, reduction in disease activity as assessed by both the investigator and patient; and by improved functional capacity as demonstrated by an increase in grip strength, and a decrease in time to walk 50 feet.

Following single oral doses of Capsules INDOMETHACIN 25 mg or 50 mg, INDOMETHACIN is readily absorbed, attaining peak plasma concentrations of approximately 1 and 2 mcg/ml, respectively, at about 2 hours. Orally administered Capsules INDOMETHACIN are virtually 100% bioavailable, with 90% of the dose absorbed within 4 hours.

Capsules INDOMETHACIN SR 75 mg are designed to release 25 mg of the medicine initially and the remaining 50 mg over approximately 12 hours (90% of dose absorbed by 12 hours). When measured over a 24 hour period, the cumulative amount and time-course of INDOMETHACIN absorption from a single Capsule

INDOMETHACIN SR are comparable to those of 3 doses of 25 mg Capsules INDOMETHACIN given at 4-6 hour intervals.

Plasma concentrations of INDOMETHACIN fluctuate less and are more sustained following administration of Capsules of INDOMETHACIN SR than following administration of 25 mg Capsules INDOMETHACIN given at 4-6 hour intervals. In multiple-dose comparisons, the mean daily steady-state plasma level of INDOMETHACIN attained with daily administration of Capsules INDOMETHACIN SR 75 mg was indistinguishable from that following Capsules INDOMETHACIN 25 mg given at 0, 6 and 12 hours daily. However, there was a significant difference in INDOMETHACIN plasma levels between the two dosage regimens, especially after 12 hours.

Controlled clinical studies in patients with osteoarthritis have shown that one Capsule INDOMETHACIN SR was clinically comparable to one 25 mg Capsule INDOMETHACIN t.i.d; and in controlled clinical studies in patients with rheumatoid arthritis, one Capsule INDOMETHACIN SR taken in the morning and one in the evening were clinically indistinguishable from one 50 mg Capsule INDOMETHACIN t.i.d.

INDOMETHACIN is eliminated via renal excretion, metabolism, and biliary excretion. INDOMETHACIN undergoes appreciable enterohepatic circulation. The mean half-life of INDOMETHACIN is estimated to be about 4.5 hours. With a typical therapeutic regimen of 25 or 50 mg t.i.d, the steady-state plasma concentrations of INDOMETHACIN are an average 1.4 times those following the first dose.

INDOMETHACIN exists in the plasma as the parent medicine and its desmethyl, desbenzoyl, and desmethyl-desbenzoyl metabolites, all in the unconjugated form. About 60% of an oral dosage is recovered in urine as medicine and metabolites (26% as INDOMETHACIN and its glucuronide), and 33% is recovered in faeces (1.5% as INDOMETHACIN).

About 90% of INDOMETHACIN is bound to protein in plasma over the expected range of therapeutic plasma concentrations.

Indications

RHEUMACIN is indicated in active stages of:

1. Rheumatoid arthritis
2. Osteoarthritis
3. Degenerative joint disease of the hip
4. Ankylosing spondylitis
5. Acute gouty arthritis

It is also included for:

Acute musculoskeletal disorders, such as bursitis, tendonitis, synovitis, tenosynovitis, capsulitis of the shoulder, sprains and strains.

Low back pain (commonly referred to as lumbago).

Fever (as a short-term adjunct to specific therapy).

Inflammation, pain, trismus and swelling following dental procedures.

Inflammation, pain and swelling following orthopaedic surgical procedures and nonsurgical procedures associated with reduction and immobilisation of fractures or dislocations.

Pain and associated symptoms of primary dysmenorrhoea.

Dosage and Administration

Dolonex* Gel

(Piroxicam)

DESCRIPTION

Dolonex, brand of piroxicam, is a member of the chemical class of nonsteroidal anti-inflammatory drugs (NSAIDs), the oxicams. Piroxicam is an amphoteric compound. It occurs as a white to off-white crystalline solid, poorly soluble in water, dilute acid, and most organic solvents. It is slightly soluble in alcohols and in aqueous alkaline solution.

Dolonex 0.5% Gel is available as a clear pale yellow gel.
Each gram of gel contains piroxicam USP 5 mg

Inert excipients include: ethyl alcohol, benzyl alcohol, propylene glycol, carbopol 940, di-isopropanolamine, hydroxyethyl cellulose, and water.

ACTIONS

Dolonex Gel is a nonsteroidal anti-inflammatory (NSAID) agent which also possesses analgesic properties. Edema, erythema, tissue proliferation, fever and pain can all be inhibited in laboratory animals by the administration of Dolonex Gel.

Acute and chronic toxicity and irritation studies have been carried out in animals. In an acute study, albino rats were given a single dermal application of 5g/kg (200 - 300 times the recommended clinical application). No deaths, toxic signs or skin irritation were observed and no gross changes were found at autopsy. A one month study was conducted in albino rats. One group received a daily application of gel to dorsal skin of 1 g per rat, another was treated with the vehicle and the third group served as untreated controls. No skin irritation was noted at the treatment sites, and no drug-related changes were observed in hemotology, laboratory chemistries, organ weight, autopsy findings or histopathology. The gel was also evaluated for primary skin irritation, eye irritation, and phototoxicity in rabbits, and for photoallergy and skin sensitization potential in guinea pigs, all according to standard established protocols. No skin reactions were found after application of 0.5% gel or the vehicle to intact rabbit skin. On abraded skin, piroxicam gel produced slight erythema and edema which was slightly greater than that following vehicle.

The anti-inflammatory and analgesic effects of Dolonex 0.5% Gel were studied in rats and guinea pigs using such standard models of pain and inflammation as carrageenin induced rat paw edema, ultraviolet erythema in guinea pigs, traumatic edema in rats, yeast induced pain in rats, croton oil induced erythema on guinea pigs abdomens, cotton pellet induced granuloma formation in rats and adjuvant induced arthritis in rats. Dolonex 0.5% Gel was comparable to indomethacin 1% gel in all of these models and was comparable to orally administered piroxicam in inhibiting inflammation in the rat paw edema model.

930201
Registered Office: Pfizer Centre, 5 Patel Estate, S V Road, Jogeshwari (W), Mumbai 400 102

On the basis of various pharmacokinetic and tissue distribution studies in rats and dogs, piroxicam 0.5% gel is continuously and gradually released from the skin to underlying muscle or synovial fluid. In addition, equilibrium between skin and muscle or synovial fluid appears to be reached rapidly, within a few hours after application.

A multiple dose study of twice-daily application of piroxicam 0.5% gel (total daily dose equivalent to 20 mg per day, piroxicam) for 14 days found that plasma levels rose slowly over the course of the treatment period and reached a value of over 200 ng/ml on the 4th day. On average, steady state plasma levels were between 300 and 400 ng/ml and mean values remained below 400 ng/ml even on the 14th day of treatment. These piroxicam levels observed at equilibrium were approximately 5% of those observed in subjects receiving similar oral dosing (20 mg daily). Elimination half-life in this study was calculated to be approximately 79 hours. In humans, the gel was well tolerated in skin sensitive volunteers.

INDICATIONS

Dolonex Gel is indicated for a variety of conditions characterized by pain, inflammation and stiffness, such as osteoarthritis (arthrosis, degenerative joint disease) of superficial joints such as the knee, post-traumatic or acute musculoskeletal disorders including tendonitis, tenosynovitis, periarthritis, sprains, strains and low back pain.

CONTRAINDICATIONS

1. Dolonex Gel should not be used in those patients who have previously shown a hypersensitivity to the gel or piroxicam in any of its dosage forms. The potential exists for cross sensitivity to aspirin and other nonsteroidal anti-inflammatory drugs.

2. Dolonex Gel should not be given to patients in whom aspirin and other nonsteroidal anti-inflammatory drugs induce the symptoms of asthma, rhinitis, angioedema or urticaria.

Dolonex* Gel

(Piroxicam)

WARNINGS

Use in Pregnancy and in Nursing Mothers: Although no teratogenic effects were seen when Dolonex was orally administered in animal testing, the safety of Dolonex use during pregnancy or during lactation has not yet been established. Dolonex inhibits prostaglandin synthesis and release through a reversible inhibition of the cyclo-oxygenase enzyme. This effect, as with other nonsteroidal anti-inflammatory agents has been associated with an increased incidence of dystocia and delayed parturition in pregnant animals when drug administration was continued into late pregnancy. Nonsteroidal anti-inflammatory drugs are also known to induce closure of the ductus arteriosus in infants.

The presence of piroxicam in breast milk has been determined during initial and long term dosing conditions (52 days). Piroxicam appeared in breast milk at about 1% to 3% of the maternal plasma concentrations. No accumulation of piroxicam occurred in milk relative to that in plasma during treatment. Dolonex gel is not recommended for use in nursing mothers as the clinical safety has not been established.

Use in Children: Dosage recommendations and indications for use in children have not been established.

PRECAUTIONS

If local irritation develops, the use of the gel should be discontinued and appropriate therapy instituted as necessary. Do not apply to the eyes, mucosal surfaces, or to any sites affected by open skin lesions, dermatoses, or infections.

ADVERSE REACTIONS

Side effects possibly related to treatment have been infrequently reported. In clinical trials the vast majority of side effects involved mild or moderate local irritation, erythema, rash, pityroid desquamation, pruritus, and related local reactions at the application site. Mild but transient skin discoloration and staining of clothing have been noted when the gel is not rubbed in completely.

OVERDOSAGE

Overdosage is unlikely to occur with this topical condition.

Dolonex* Gel

(Piroxicam)

DOSAGE AND ADMINISTRATION

This product is intended for external use only. No occlusive dressing should be employed. Rub in the gel leaving no residual material on the skin.
A dosage of one gram, equivalent to 5 mg of piroxicam (corresponding to approximately 3 cm or 1¼") should be applied to the affected site three or four times per day. Therapy should be reviewed after 4 weeks.

SUPPLY

Dolonex 0.5% Gel: Tubes of 15g and 50g.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No.
09/446,109 by The
University of Queensland

STATUTORY DECLARATION UNDER RULE 132

I, Dr Stephen Maxwell Taylor of 17 Perdita Street, Bellbird Park, in the State of Queensland 4300, Commonwealth of Australia, do solemnly and sincerely declare as follows:

1. I am an Associate Professor in the Department of Physiology and Pharmacology at The University of Queensland. I am also an inventor in respect of United States Patent Application No. 09/446,109.

2. My relevant experience is attached hereto as Appendix A. A copy of my curriculum vitae is now shown to me, and is annexed hereto as Exhibit SMT-1.

3. I have read and understood the Office Action dated 18 December 2003 issued in respect of this application.

4. In this Office Action, the Examiner has raised an objection under Section 112 first paragraph that the specification does not provide an enabling disclosure in respect of a method of treating an inflammatory condition, included a method of treating inflammatory arthritis, comprising the step of administering an effective amount of a compound of the invention to a mammal in need thereof. I understand that the Examiner

has formed this opinion on the basis that while the specification discloses certain experimental models, and refers to treatment of rheumatoid arthritis. The Examiner does not consider that these model systems would be regarded in the art as being reasonably predictive of efficacy in other inflammatory arthritides, or in inflammatory conditions other than inflammatory arthritis.

5. I believe that the Examiner is incorrect in taking this position, because I consider that in fact the model systems described in the specification are very well accepted as being generally predictive of anti-inflammatory efficacy, not only in the treatment of rheumatoid arthritis but in other inflammatory conditions. Moreover, I consider that efficacy in the treatment of rheumatoid arthritis is regarded as being reasonably predictive of efficacy in other inflammatory arthritides. I do not consider that any further inventive effort would be required in order to demonstrate efficacy of the compounds of the invention in such conditions.

6. In fact, my colleagues and I have carried out further experimentation using assays and model systems which are well-known in the art, and have not required any further inventive effort in order to do so. This is discussed further below.

7. Using *in vitro* assays, such as those described on page 26, lines 1 to 19, and line 21 to page 27, line 2 of the specification, and the rat carrageenin paw oedema model and rat adjuvant arthritis model described in Example 8 of the specification, we have also demonstrated that the compounds of the invention have anti-inflammatory and anti-arthritic activity. The data obtained using the antigen-induced arthritis model have already been provided to the Examiner in manuscript form with our first response dated 21 April 2000. These results have now been published in *Arthritis and Rheumatism* 2002 46:2476-2485. A copy of this paper is now shown to me and annexed hereto as Exhibit SMT-2.

8. I consider that it is self-evident that it is standard practice in the art to use the cheapest and simplest experimental model first, and to progress a candidate agent to more complex, expensive and time consuming models only if the preliminary screening tests are successful. Both carrageenin-induced inflammation and antigen-induced

arthritis are often thought to be more successful in the rabbit than in the rat, but the rat is a much cheaper animal to use. Antigen-induced arthritis requires prior sensitization, and thus is more time-consuming than carrageenin-induced inflammation. Collagen-induced arthritis is also a notoriously variable model of human rheumatoid arthritis, and expression of the disease is highly variable in rodents, which are the preferred species. Practitioners in the art emphasise the difficulty of using commercial supplies of the antigen, which is associated with poor expression of the disease, and recommend the raising and purifying of collagen antigen from cultured bovine nasal epithelia in the investigators' own laboratory. This increases the costs and expertise required, and so restricts the use of this model. Carrageenin-induced footpad oedema is quick and easy to induce, and can readily be induced in rats; thus it is often used in preliminary screening.

9. The specification of the present application discloses *in vitro* assays for testing candidate anti-inflammatory agents for activity using a suitable surrogate marker. For example, a receptor-binding assay is described at page 26 lines 1 to 19 of the present specification, and an enzyme assay is described at page 26 line 21 to page 27 line 2.

10. I consider that it is a matter of routine then to proceed to *in vivo* assays such as the carrageenin paw oedema and adjuvant arthritis models described at page 27 line 4 to page 28 line 34 of the specification. Similarly, I consider that because the antigen-induced arthritis model is so well known it would require no further inventive activity to test the candidate agent in this model.

11. I emphasise that the *in vitro* assays described in the specification and the rat paw oedema model are general models useful for testing anti-inflammatory activity, and are not restricted to assessment of efficacy in the treatment of arthritis. They are therefore useful as preliminary tests for efficacy in *any* inflammatory condition.

12. The antigen-induced arthritis model shares many histological similarities with human rheumatoid, as well as with other arthritides, including hyperplasia of the synovial membrane, mononuclear cell infiltration, acute and chronic phases, pannus formation, and secondary cartliage erosion. The model also shares the joint swelling and correlated mobility impairment seen in acutely inflamed human arthritic joints. Because

of the antigen-antibody nature of disease induction, the model has also been proposed as a model of reactive arthritis. The immuno-inflammatory basis of the pathology in the antigen-induced arthritis model also mimics several features of human inflammatory arthrides, including synovial and systemic elevations in inflammatory cytokines, and the necessity for immune and inflammatory cell involvement.

13. The antigen-induced arthritis model also shows numerous clinical and pathological similarities with psoriatic arthritis and reactive arthritis, and consequently is considered in the art to be a valid and useful animal model of human inflammatory arthritis. Thus, these features of antigen-induced arthritis in various experimental animals make this model useful as a model for ascertaining the effectiveness of new disease therapies.

14. The antigen-induced arthritis model is a well-established experimental model of arthritis. The model is also highly responsive to anti-inflammatory and immune-based therapies, with significant efficacy shown by ibuprofen, indomethacin, prednisolone, infliximab, leflunomide, methotrexate, and several other agents which are used with success in treating human RA and other arthritides. Copies of these articles are now shown to me, and are annexed hereto as Exhibits SMT-3, SMT-4 and SMT-5.

15. For example, psoriatic arthritis is an inflammatory disease which affects joints, ligaments, tendons and sometimes the spine. Although psoriatic arthritis is associated with psoriasis, and occurs in a subset of patients with psoriasis, these two conditions are considered to be distinct diseases, which may have different pathophysiologies. According to the American College of Rheumatology, non-steroidal anti-inflammatory drugs (NSAIDs) are the initial treatment for arthritis symptoms in patients with psoriatic arthritis, an inflammatory arthritis. Consequently screening methods suitable for testing of anti-inflammatory drugs such as NSAIDs are suitable for testing candidate agents for treatment of this condition. Ankylosing spondylitis is a form of chronic inflammatory arthritis which most often affects the spine, and is treated in the same way as rheumatoid arthritis, with the same armory of drugs, including NSAIDs; Inflixamal (Remicade™), an antagonist of tumour necrosis factor (TNF), a general

mediator of inflammatory responses, is approved for use in the treatment of rheumatoid arthritis. Psoriatic arthritis and ankylosing spondylitis are sometimes referred to as spondyloarthropathies, because they affect areas around the joint where ligaments and tendons attach to bone (enthesitis) rather than the lining of the joint (synovium). However, the underlying pathological mechanisms are thought to be similar.

16. I therefore believe that a person of ordinary skill in the art would, once in possession of the present specification, have a reasonable expectation that the compounds of the invention would be useful in the treatment of numerous inflammatory conditions, especially several inflammatory arthritides, and most notably rheumatoid arthritis. Moreover, such a person would readily be able to test the compounds of the invention in well-established experimental models, without the need to exercise any further inventive effort. Accordingly, the specification provides sufficient support for claims that a method of treating inflammatory arthritis, comprising the step of administering an effective amount of a compound of the invention to a mammal in need thereof.

17. These tests are well known to have broad application. Although the present specification specifically describes the application of the invention to rheumatoid arthritis, a person skilled in the art would clearly understand that the invention has application to inflammatory and arthritic conditions in general, as well as inflammatory arthritic conditions, such as rheumatoid arthritis.

18. We have carried out further experiments using the antigen-induced arthritis model in a study of the role of the complement system in rheumatoid arthritis. The results of these experiments have been set out in a draft manuscript, a copy of which is now shown to me, and is annexed hereto as Exhibit SMT-6.

19. This manuscript describes antigen-induced arthritis as being an established model of RA that involves stimulation of T-lymphocyte reactivity against the immunizing antigen. This model is induced by the immunization of animals with a protein antigen (methylated bovine serum albumin, ovalbumin or fibrin) and an adjuvant, followed by the intra-articular injection of the same antigen. This results in an immune-complex mediated inflammatory response, characterised by chronic synovitis, which is

localised to the injected joint. The ability to localise inflammation to the antigen-injected joint only (monoarticular arthritis) allows for an internal control in the contra-lateral joint. Many of the disease pathologies in this model mimic those seen in human rheumatoid arthritis (Table 1), having both acute and chronic phases of disease. There is also the capacity to induce subsequent flare-ups, which are commonly seen in rheumatoid arthritis, through the re-injection of the antigen.

20. Moreover, the US Food and Drug Administration's Center for Biologics Evaluation and Research (CBER) provides guidance for industry on "Clinical Development Programs for Drugs, Devices and Biological Products for the Treatment of Rheumatoid Arthritis (RA)" on its website at http://www.fda.gov/cber/gdlns/rheumcln.htm. This is an extremely detailed set of guidelines, and Section III at page 6 sets out considerations in rheumatoid arthritis product development, including the selection of appropriate *in vitro* models (animal or human services) and *in vivo* animal models for screening potentially active agents. Page 8 states that collagen-induced arthritis is often considered to be a suitable model; this is a form of antigen-induced arthritis. The rat carrageenin-induced acute model of inflammation is stated to be useful in assessing anti-inflammatory activity, and it is also stated that most of the animal models which involve inflammation in the paw may be used for measuring antiphlogistic (i.e. anti-inflammatory) action of a drug. This is the carageenin-induced footpad inflammation model which is described in the present specification. Copies of the relevant pages from the US Food and Drug Administration's Center for Biologics Evaluation and Research website are now shown to me, and are annexed hereto as Exhibit SMT-7.

21. A cursory search of the PubMed database reveals 25 pages of titles of publications on carrageenin-induced arthritis, and 138 pages of titles of publications on antigen-induced arthritis. Clearly these are both extremely widely-used models.

22. Once the anti-inflammatory and anti-arthritic activity of the compounds of the invention had been demonstrated as described in the present specification, it was a matter of mere routine trial and error experimentation to formulate a method of treating

inflammatory arthritis using the compounds of the present invention, and to test this method using well-accepted animal models, as discussed above. These *in vivo* experiments showed that the method was indeed effective.

23. In fact the results were so favourable that the lead compound of the invention has progressed to human clinical trials in the treatment of rheumatoid arthritis, using oral administration, and of psoriasis, using topical administration. The results of these trials have been favourable, and a summary is now shown to me and is annexed hereto as SMT-8. Please note that this information is to be treated as commercial-in-confidence.

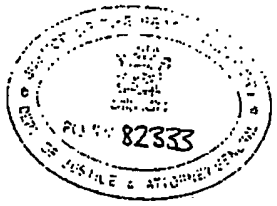
24. I therefore consider that a person of ordinary skill in the art would, once in possession of the present specification, have a reasonable expectation that the compounds of the invention would be useful in the treatment of numerous inflammatory conditions, especially several inflammatory arthritides, and most notably rheumatoid arthritis. Moreover, such a person would readily be able to test efficacy of the compounds of the invention for this purpose in well-established experimental models, without the need to exercise any further inventive effort. Accordingly, I consider that the specification provides an enabling disclosure of a method of treating inflammatory arthritis, comprising the step of administering an effective amount of a compound of the invention to a mammal in need thereof.

And I make this solemn Declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by the Act for the making of false statements in Statutory Declarations, conscientiously believing the statements contained in this Declaration to be true in every particular.

DECLARED at Brisbane this 12 day of MAY 2004

.....
Stephen Maxwell Taylor

Before me: .....



A person empowered to witness Statutory Declarations under the laws of the State of Queensland, Commonwealth of Australia

APPENDIX A

I have worked in the field of inflammation for more than 20 years, beginning with post-doctoral studies at the Washington State University, WA, USA. There I studied the role of inflammatory mediators in causing pulmonary inflammation. I then moved to San Diego in 1998 to Immunetech Pharmaceuticals, where I directed the search for new kinds of anti-inflammatory drugs and began work on the C5a antagonist project. In 1990 I moved to the University of Queensland and continued the development of C5a receptor antagonists, culminating in their discovery in 1997. Since then I have continued this work, and published extensively on the efficacy of these new drugs in numerous animal models of inflammatory disease where complement and C5a have been implicated as pathogenic factors. Details of this background and list of publications are detailed in my curriculum vitae.

12/05/04

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-1

This is Exhibit SMT-1 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Toni Russo

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

CURRICULUM VITAE

Steve M. Taylor

Personal:

Place of birth:	England
Citizenship:	British/Australian

Education:

B.Sc. (Hons.1), 1971	University of Sydney, Australia
Ph.D. (Pharmacology), 1978	University of Sydney, Australia

Post-Doctoral Experience:

1978-1982	Research Associate, Dept. of Pharmacology, School of Medicine, Oregon Health Sciences University, Portland, OR, U.S.A.
1982-1983	Research Fellow, Pulmonary Research Laboratory, Dept. of Medicine, St. Paul's Hospital, Vancouver, B.C., Canada
1983-1988	Research Assistant Professor, Dept. of Veterinary and Comparative Anatomy, Physiology and Pharmacology, & Dept. of Veterinary Microbiology & Pathology,Washington StateUniversity, Pullman, WA, U.S.A.
1988-1990	Senior Scientist, Immunetech Pharmaceuticals, San Diego, CA , U.S.A.
1990-1993	Lecturer in Pharmacology, University of Queensland Brisbane, Qld 4072, Australia
1994-1999	Senior Lecturer, University of Queensland Brisbane, Qld 4072, Australia
11/1999	Director of Pharmacology, *Promics* Pty Ltd
2000-	Reader in Pharmacology, Univ. Qld
2001-	Chief Scientist, Promics P/L

Membership in Professional Societies:

American Society for Pharmacology and Experimental Therapeutics
Leukocyte Society
Australian Society For Clinical and Experimental Pharmacology and Toxicology
Australian Society for Cytokines, Inflammation and Leukocytes

Teaching:

1970-75	Demonstrator in Pharmacology, University of Sydney
1980-81	Visiting Lecturer in Pharmacology, Oregon Health Sciences University, Portland, Oregon, USA
1983-84	Visiting Lecturer in Pharmacology, Department of Pharmacology, College of Veterinary Medicine, Washington State University, Pullman, WA,.USA
1990-	Lecturer/Senior Lecturer/Reader in Pharmacology, University of Queensland

PUBLICATIONS

1. Malor, R., Griffin, C.J., and Taylor, S.M. Innervation of the blood vessels in the guinea pig atria. *Cardiovasc. Res.* 7: 95-104, 1973.

2. Malor, R., Taylor, S.M., Chesher, G.B. and Griffin, C.J. The intramural ganglia and chromaffin cells in quinea pig atria: an ultrastructural study. *Cardiovasc. Res.* 8: 731-744, 1974.

3. Taylor, S.M., Rodgers, R.M., Lynn, R.K., and Gerber, N. The seminal excretion, plasma half-life, tissue distribution and metabolism of naltrexone in the rabbit. *J. Pharmacol. Exp. Ther.* 213: 289-299,1980.

4. Dickinson, R.G., Taylor, S.M., Kaufman, S.N., Rodgers, R.M., Gerber, N., and Baughman, W.J. Nonlinear elimination and choleretic effect of valproic acid in the monkey. *J. Pharmacol. Exp. Ther.* 213: 38-48, 1980.

5. Rogers, R.M., Taylor, S.M., Dickinson, R.G., Ilias, A.M., Lynn, R.K., and Gerber, N. Metabolism and choleretic effect of naltrexone in the isolated perfused rat liver. *Drug. Metab. Dispos.* 8: 390-396, 1980.

6. Koga, Y., Downes, H., and Taylor, S.M. Direct and indirect actions of dopamine on tracheal smooth muscle. *Nauyn Schmiedeberg's Arch. Pharmacol.* 315: 15-20, 1980.

7. Ilias, A.M., and Taylor, S.M. The seminal excretion, blood elimination, tissue distribution and metabolism of L-alpha-acetylmethadol in the rabbit. *Res. Commun. Subst. Abuse* 3: 443-452, 1982.

8. Downes, H., and Taylor, S.M. A potent, structurally specific stimulant action of procaine in bullfrog lung. *Comp. Biochem. Physiol.* 72(C): 39-43, 1982.

9. Taylor, S.M., and Downes, H. Bronchodilator mechanisms in bullfrog lung: differences in response to isoproterenol, theophylline and papaverine. *J. Pharmacol. Exp. Ther.* 223: 359-367, 1982.

10. Downes, H., and Taylor, S.M. Distinctive pharmacological profile of a nonadrenergic inhibitory system in bullfrog lung. *Br. J. Pharmacol.* 78: 339-351, 1983.

11. Taylor, S.M., Downes, H., Hirshman, C.A., Peters, J.E., and Leon, D. Pulmonary uptake of mannitol as an index of changes in lung epithelial permeability. *J. Appl. Physiol.* 55: 614-618, 1983.

12. Taylor, S.M., and Downes, H. Effect of stimulation of nonadrenergic inhibitory nerves on cyclic nucleotide levels in bullfrog lung. *Comp. Biochem. Physiol.* 77(C): 311-313, 1984.

13. Taylor, S.M., Pare, P.D., and Schellenberg, R.R. Cholinergic and nonadrenergic mechanisms in human and guinea pig airways. *J. Appl. Physiol.* 56: 958-965, 1984.

14. Armour, C.L., Lazar, N.M., Schellenberg, R.R., Taylor, S.M., Chan, N., Hogg, J.C., and Pare, P.D. A comparison of *in vivo* and *in vitro* human airway reactivity to histamine. *Am. Rev. Respir. Dis.* 129: 907-910, 1984.

15. Taylor, S.M., Bennett, G., Abbott, L.C., and Finnell, R.H. Anticonvulsant sensitivity in the mutant mouse, quaking. *European J. Pharmacol.* 118: 163-170, 1985.

16. Taylor, S.M., Pare, P.D., Armour, C.L., Hogg, J.C. and Schellenberg, R.R. Airway reactivity in Chronic Obstructive Pulmonary disease: Failure of *in vivo* methacholine responsiveness to correlate with cholinergic, adrenergic, or non-adrenergic responses *in vitro*. *Am. Rev. Resp. Dis.* 132: 30-35, 1985.

17. Taylor, S.M., Liggitt, H.D., Laegreid, W.W., Silflow, R., and Leid, R.W. Arachidonic acid metabolism in bovine alveolar macrophages. Effect of calcium ionophore on lipoxygenase products. *Inflammation.* 10: 157-165, 1986.

18. Downes, H., Edelstein, R.A., and Taylor, S.M. Mechanisms of intrinsic tone in bullfrog lung; relaxant effects of indomethacin, ouabain, and potassium. *Brit. J. Pharmacol.* 87: 579-586, 1986.

19. Leid, R.W., Taylor, S.M., Heidel, J.R., Laegreid, W.W., Liggitt, H.D., Potter, K.A., Silflow, R., and Breeze, R.G. Arachidonate metabolites from bovine alveolar and peripheral blood neutrophils . *Proceedings 14th World Congress of Diseases of Cattle.* 1: 549-554, 1986

20. Husain, A., Pajka, S.F., Taylor, S.M., and Speth, R.C. Biological actions of monoiodo-angiotensin II. *J. Pharmacol. Exp. Ther.* 239: 71-77, 1986.

21. Finnell, R.H., Mohl, V.R., Bennett, G.B., and Taylor, S.M. The role of epoxidation in carbamazepine-induced teratogenesis. *Teratogenesis Mutagenesis and Carcinogenesis.* 6: 393-401, 1986.

22. Lyles, W.C., Taylor, S.M., Finnell, R.H., Lai, H., and Nathanson, N. Decreased muscarinic acetylcholine receptor number in the CNS of the tottering (tg/tg) mouse. *J. Neurochem.* 46: 977-982, 1986.

23. Willow, M., Taylor, S.M., Catterall, W.A., and Finnell, R.H. Down regulation of sodium channels in nerve terminals of spontaneously epileptic mice. *Cellular and Molecular Neurobiology.* 6: 213-220, 1986.

24. Taylor, S.M., and Finnell, R.H. Effect of quinidine and tetrodotoxin on non-adrenergic neurones in guinea-pig trachealis muscle. *Comp. Biochem. Physiol.* 86C: 11-15, 1987.

25. Pare, P.D., Armour, C.A., Taylor, S.M., Mullen, B., Moreno, R., Hogg, J.C. and Schellenberg, R.R. Airway hyperreactivity in COPD. Cause or effect, an *in vitro-in vivo* comparison. *Chest* 91: 41S-44S, 1987.

26. Taylor, S.M., Laegreid, W.W., Heidel,J., Straub, K., Liggitt, H.D., Silflow, R., Breeze, R.G., and Leid, R.W. Arachidonic and eicosapentaenoic acid metabolism in bovine peripheral blood neutrophils and platelets: effect of calcium ionophore. *J. Leukocyte. Biol. 42:* 253-262, 1987.

27. Finnell, R.H., Shields, H., Taylor, S.M., and Chernoff, G.F.. Strain differences in phenobarbital-induced teratogenesis in mice. *Teratology.* 35: 177-185, 1987.

28. Laegreid, W.W., Taylor, S.M., Liggitt, H.D., Straub, K., Silflow, R., Breeze, R.G., and Leid, R.W. Production of lipoxygenase metabolites of eicosapentaenoic acid by bovine alveolar macrophages *in vitro. Inflammation* 12: 503-514, 1988.

29. Finnell, R.H., Abbott, L.C., and Taylor, S.M. The fetal hydantoin syndrome: answers from a mouse model. *Reproductive Toxicology.* 3:127-133, 1989.

30. Laegreid, W.W., Taylor, S.M., Englen, M.D., Silflow, R.M., Liggitt, H.D., and Leid, R.W. Stimulus-selective production of cyclo-oxygenase and lipoxygenase metabolites of arachidonic acid by bovine alveolar macrophages. *Inflammation* 13: 233-244, 1989.

31. Laegreid, W.W., Liggitt, H.D., Silflow, R.M., Evermann, J.R., Taylor, S.M., and Leid, R.W. Reversal of virus-induced alveolar macrophage bactericidal dysfunction by cyclo-oxygenase inhibition *in vitro. J. Leukocyte Biol.* 45: 283-292, 1989

32. Laegreid, W.W., Taylor, S.M., Liggitt, H.D., Evermann, J., Silflow, R and R.W. Leid. Virus-induced enhancement of arachidonate metabolism by bovine alveolar macrophages *in vitro. J. Leukocyte Biol.* 45: 293-300, 1989.

33. Englen, M.D., Taylor, S.M., Laegreid, W.W., Liggitt, H.D., Silflow, R.M. and Leid, R.W. Stimulation of arachidonic acid metabolism in silica-exposed alveolar macrophages. *Exp. Lung Research.* 15: 511-526,1989.

34. Heidel, J.R., Taylor, S.M., Laegreid, W.W., Silflow, R.M., Liggitt, H.D., and Leid, R.W. Characterisation of arachidonic acid metabolism, superoxide production, and

bacterial killing in bovine circulating neutrophils and elicited alveolar neutrophils. *J. Leukocyte Biol.* 46: 41-50, 1989

35. Heidel, J.R., Taylor, S.M., Laegreid, W.W., Silflow, R.M., Liggitt, H.D. and Leid, R.W. *In vivo* chemotaxis of bovine neutrophils by 5-lipoxygenase metabolites of arachidonic and eicosapentaenoic acid. *Am. J. Pathology.* 134: 671-676, 1989.

36. Silflow, R.M., Foreyt, W.J., Taylor, S.M., Laegreid, W.W., Liggitt, H.D. and R.W. Leid. Comparison of pulmonary defense mechanisms in bighorn and domestic sheep. *J. Wildlife Diseases* 25: 514-520, 1989

37. Heidel, J.R, Sassenfeld, C.R., Maliszewdki, R.M. Silflow, Baker, P.E, Taylor, S.M. and R.W. Leid. Functional studies of bovine alveolar neutrophils elicited with recombinant bovine IL-1β. *J. Immunol.* 144: 1037-1041, 1990

38. Taylor, S.M., Laegreid, W.W., Englen, M.D., Dani, G., Silflow, R., Liggitt, H.D. and R.W. Leid. Influence of extracellular calcium on arachidonic acid metabolism in alveolar macrophages. *J. Leukocyte Biol.* 48: 502-511, 1990

39. Englen, M.D., Taylor, S.M., Laegreid, W.W., Silflow, R., Banks, K. and R.W. Leid. Arachidonic acid metabolism in caprine alveolar macrophages. *Inflammation* 14: 239-245, 1990

40. Englen, M.D., Taylor, S.M., Laegreid,W.W., Silflow, R.M. and R.W. Leid. Effects of different silicas on arachidonate metabolism in alveolar macrophages. *Exp. Lung Res.* 16: 691-709, 1990

41. Heidel, J.R., Taylor, S.M., Silflow, R.M. and R.W. Leid. Characterisation of arachidonic acid metabolism, superoxide production and bacterial killing by bovine alveolar neutrophils elicited with LTB4 and zymosan activated plasma. *Inflammation* 1991, 15: 31-42

42. Silflow, R.M, Taylor, S.M., Laegreid, W.W., Liggitt, H.D. and R.W. Leid. Comparison of arachidonic acid metabolism by alveolar macrophages in bighorn and domestic sheep. *Inflammation* 14: 43-54, 1991

43. Englen, M.D., Taylor, S.M., Laegreid, W.W., Silflow, R. M. and R.W. Leid. Diminished arachidonic acid release by bovine alveolar macrophages exposed to surface-modified silica. *Am. J. Respir. Cell Mol. Biol.* 6: 527-534, 1992

44. Ember, J.A., Sanderson, S. D., Taylor, S.M., Kawahara, M., and T.E.Hugli. Biologic activity of synthetic analogs of C5a anaphylatoxin. *J. Immunol.* 148: 3165-3173, 1992

45. Taylor, S.M., Heron, A.E., Cannell, G.R. and T.H. Florin. Pressor effect of ethanol in the isolated, perfused human placental lobule. *Eur. J. Pharmacol.* 270: 371-374, 1994

46. Taylor, S.M., Finch, A.M., Heron, A.E., Brown, L.C. and T.H. Florin. Reversibility of tachyphylaxis to C5a in guinea-pig tissues, the perfused human placental lobule and the umbilical artery. *Inflammation* 18: 645-657, 1994

47. Sanderson, S.D., Ember, J.A., Kirnarsky, L., Sherman, S.A, Finch, A. M and S.M. Taylor. Decapeptide agonists of human C5a: The relationship between

conformation and spasmogenic and platelet aggregatory activites. *J. Med. Chem.* 37: 3171-3180, 1994

48. Shiels, I.A., Bowler, S.D. and S.M. Taylor. Airway smooth muscle proliferation in asthma: the potential of vascular leakage to contribute to pathogenesis. *Med. Hypotheses.* 45: 37-40, 1995

49. Finch, AM, Heron AE, Tolhurst SL, Florin, TH, Sanderson SD & SM Taylor. The effect of C5a and U46619 on the isolated, perfused human placental lobule: development of a method for the on-line estimation of tissue fluid accumulation J. *Pharmacol Toxicol Methods.* 34: 133-141, 1995

50. Sanderson SD, Kirnarsky L, Sherman S, Ember JA, Finch AM & S M Taylor. Decapeptide agonists of human C5a: the relationship between conformation and neutrophil response, *J. Med. Chem.* 38: 3669-3675, 1995

51 Shiels IA, Bowler, SD and SM Taylor. Homologous serum increases fibronectin expression and cell adhesion in airway smooth muscle cells. *Inflammation* 20: 373-388, 1996 7

52. Kawatsu, R, Sanderson, SD, Blance, I, Kendall, N, Finch, AM, Taylor, SM and D. Colcher. Conformationally biased analogues of human C5a mediate changes in vascular permeability. *J. Pharmacol. Exp. Therap.* 278: 432-440, 1996 11

53 Finch, AM, Vogen, SM, Sherman, SA, Kirnarsky, L, Taylor, SM and SD Sanderson. A biologically active conformer of the effector region of human C5a and the modulatory effects of N-terminal receptor binding determinants on activity. *J. Med Chem.* 40: 877-884, 1997 22

54 Tempero, RM, Hollingsworth, MA, Burdick, MD, Finch, AM, Taylor SM, Vogen SM, Morgan EL and Sanderson, SD Molecular adjuvant effects of a conformationally biased agonist of human C5a anaphylatoxin *J. Immunol.* 158: 1377-1382, 1997 15

55 Shiels, IA, Zhang, S, Ambler, J and SM Taylor. Homologous serum induces phenotypic change and fibronectin expression in cultured retinal epithelial cells *Med. Hypotheses.* 50: 113-117, 1998. *6*

56 Wong, AK, Finch, AM, Pierons, G, Taylor, SM and Fairlie, DP. Molecular probes for G-protein coupled receptors. Conformationally constrained antagonists derived from the C-terminus of human plasma protein C5a. *J. Med. Chem.* 41: 3417-3425, 1998 20

57 Zhang, S., I. A. Shiels, J. Ambler and S. M. Taylor. Pirfenidone reduces fibronectin synthesis by cultured human RPE cells. *Aust. N. Z. J. Ophthalmol.* 26, 574-576, 1998. 5

58 Vogen,SM, Finch, AM, Wadi, K,Thatcher, J, Monk, PN, SM Taylor & Sanderson, SD. The role of the amino terminal end of decapeptide agonists of C5a in receptor binding and activation: Nature of the interaction of position 68 of C5a and Glu199 of the C5a receptor. *Peptide Res.* 53: 8-17, 1999

59 Shiels, IA, SD Sanderson &SM Taylor. A multi-species, arterially-perfused eye model of uveitis to test a novel anti-inflammatory agent, pirfenidone. *Aust. Vet. J.* 77: 100-104, 1999.

60 Short, A, Wong, AK, Finch, AM, Haaima, G, Shiels, IA, Fairlie, DP & SM Taylor. Effects of a new C5a receptor antagonist on C5a- and endotoxin-induced neutropenia in the rat. *Br. J. Pharmacol*. 126: 551-554, 1999 25

61 Paczkowski NJ, Finch, AM, Whitmore,J, Short, AJ, Wong, AK, Monk, PN, Cain SA, Fairlie, DP and SM Taylor Pharmacological characterisation of antagonists of the C5a receptor *Br J Pharmacol* 128: 1461-1466, 1999. 30

62 Finch, AM, Wong, AK, Paczkowski, NJ, Wadi, SK, Craik, D, Fairlie, DP and S. M. Taylor. Low molecular weight peptidic and cyclic antagonists of the C5a receptor *J. Med Chem.* 42: 1965-1974, 1999 34

63 Wong, A.K.; Taylor, S. M.; Fairlie, D. P. Development of C5a receptor antagonists *Investigational Drugs* 2: 686-693, 1999

64 Shiels, IA, Bowler SD and SM Taylor The effects of salbutamol, beclomethasone and dexamethasone on growth and fibronectin expression by cultured airway smooth muscle cells. *Inflammation* 23: 321-331, 1999 8

65 Short, AJ, Paczkowski, NJ, Vogen, SM, Sanderson SD and S M Taylor. Response-selective C5a agonists: differential effects on neutropenia and hypotension in the rat. *Br. J. Pharmacol.* 128: 511-514, 1999. 10

66 Zhang, S, Shiels IA & SM Taylor. Homologous serum stimulated fibronectin synthesis in human retinal pigmented epithelial cells. *Aust. N. Z. J. Ophthalmol* 27: 247-249, 1999

67 Vogen SM, Finch AM, Wadi SK, Thatcher J, Monk PN, Taylor SM, Sanderson SD The influence of Lys68 in decapeptide agonists of C5a on C5a receptor binding, activation and selectivity *J Peptide Res.* 53: 8-17 1999 9

68 Shiels, IA, Taylor, SM and DP Fairlie. Targeting vascular leakage and structural change in chronic inflammatory diseases. *Med Hypotheses* 54: 193-197, 2000 3

69 Ulrich, JT, Cieplak, W, Paczkowski NJ, Taylor SM and SD Sanderson. Induction of an antigen-specific CTL immune response by a conformationally biased agonist of human C5a anaphylatoxin as a molecular adjuvant *J. Immunol.* 164: 5492-5498, 2000 5

70 Haynes DR, Harkin, DJ, Bignold, LP, Hutchens, M, Taylor, SM. & DP Fairlie. Inhibition of C5a-induced neutrophil chemotaxis and macrophage cytokine production *in vitro* by a new C5a receptor antagonist. *Biochem. Pharmacol.* 60: 729-733, 2000 17

71 Strachan, AJ, Woodruff, TM Haaima, G, Fairlie, DP and SM Taylor The role of the complement system in ischemia-reperfusion injury. Inhibition of the reverse passive Arthus reaction and endotoxic shock in rats by a small molecule C5a receptor antagonist. *J. Immunol.* 164: 6560-6565, 2000 34

72 Taylor, SM & DP Fairlie. Regulators of the anaphylatoxin C5a. *Expert Opinion in Therapeutic Patents.* 10: 449-458, 2000 3

73 Shiels IA, Taylor SM, Fairlie DP Cell phenotype as a target of drug therapy in chronic inflammatory diseases Med Hypotheses 54 : 193-197, 2000 3

74 Woodruff TM, AJ Strachan, SD Sanderson, P N Monk, AK Wong,† DP Fairlie and S M Taylor. Species variance in the binding affinity of new low molecular weight antagonists and agonists of the C5a receptor *Inflammation*, 25 :171-177, 2001 9

75 Cain S. A.; Woodruff, T. M.; Taylor, S. M.; Fairlie, D. P.; Monk, P. N. Mutations in the first extracellular loop of the human C5a receptor differentially affect responses to agonists and antagonists *Biochem. Pharmacol.* 61:1571-9, 2001 6

76 Stocks, SZ, Taylor, SM and I.A. Shiels. TGF-β_1 induced α-smooth muscle actin expression and fibronectin synthesis in cultured human retinal pigment epithelial cells *Clin Experiment Ophthalmol.* 29:33-7, 2001 3

77 Taylor SM, Sherman SA, Kirnarsky L and SD Sanderson Development of response-selective agonists of human C5a anaphylatoxin: conformational, biological, and therapeutic considerations. *Curr Med Chem* 8:675-84, 2001 3

78 Miric G, Dallemagne C, Endre Z, Margolin S, Taylor SM, Brown L. Reversal of cardiac and renal fibrosis by pirfenidone and spironolactone in streptozotocin-diabetic rats. *Br J Pharmacol.* ;133:687-94, 2001. 18

79 Strachan, AJ, IA Shiels, RC Reid, DP Fairlie, SM Taylor Inhibition of immune-complex mediated dermal inflammation following either oral or topical administration of a small molecule C5a receptor antagonist. *Br J Pharmacol* 134: 1778-1786, 2001 14

80 Vogen SM, Paczkowski NJ, Kirnarsky L, Short A, Whitmore JB, Sherman SA, Taylor SM, Sanderson SD. Differential activities of decapeptide agonists of human C5a: the conformational effects of backbone N-methylation. *Int Immunopharmacol.* 1:2151-62, 2001.

81 Mirkovic S, AL Seymour, A Fenning, A Strachan, S B Margolin, S M Taylor and L Brown Selective reversal of cardiac fibrosis by pirfenidone and amiloride in DOCA-salt hypertensive rats *Br J Pharmacol* 135; 961-968, 2002 7

82 Arumugam TV, IA Shiels, S B Margolin, SM Taylor and L Brown Pirfenidone attenuates ischaemia-reperfusion injury in the rat small intestine *Clin. Exp. Physiol. Pharmacol.* 29; 996-1000, 2002 1

83 Arumugam, TV, IA. Shiels, TM. Woodruff, RC Reid, DP. Fairlie and SM. Taylor. A small molecule C5a receptor antagonist protects the rat small intestine against ischemia-reperfusion injury. *J. Surg. Res.* 103: 260-267, 2002 16

84 Woodruff TM, Strachan AJ, Dryburgh, N, Shiels IA, Reid RC, DP. Fairlie, and SM Taylor Anti-arthritic activity of an orally active C5a receptor antagonist against antigen-induced monoarticular arthritis in the rat. *Arthritis and Rheumatism* 46: 2476-2485, 2002 6

85 ArumugamTV, Shiels IA, Strachan AJ, Abbenanate G, Fairlie DP and SM. Taylor A small molecule C5a receptor antagonist protects kidneys against ischemia-reperfusion injury in rats. *Kidney International* 63:134-42, 2003

86 Harkin, DW, BB Rubin, SM Taylor, AD Romaschin, TF Lindsay Complement C5a Receptor Antagonist Attenuates Multiple Organ Injury in a Model of Ruptured Abdominal Aortic Aneurysm *J. Vasc. Surg.* 39: 196-206, 2004

87 Arumugam, T, Arnold,N , Newman,M Reid, RC, Hansford, K, Fairlie, DP Shiels, IA and SM Taylor. Comparative protection against rat intestinal reperfusion injury by a new inhibitor of $sPLA_2$, COX-1 and COX-2 selective inhibitors, and an LTC_4 receptor antagonist. *Br J Pharmacol.* 140:71-80, 2003

88 Woodruff TM, Arumugam, T, Shiels IA, Reid RC, DP. Fairlie, and SM Taylor A potent C5a receptor antagonist inhibits markers of local and remote organ injury following tourniquet-induced limb ischemia-reperfusion in rats. *J. Surg. Res.* 116: 81-90, 2004

89 Woodruff, T. M.; Arumugam, T. V.; Shiels, I. A.; Reid, R. C.; Fairlie, D. P.; Taylor, S. M. A Potent C5a Receptor Antagonist Inhibits and Reverses Disease Pathology in a Rat Model of Inflammatory Bowel Disease, *J. Immunol.* 171: 5514-5520, 2003.

90 Reid, RC, Abbenante, G, Taylor, SM and DP Fairlie A Convergent Solution Phase Synthesis of the Macrocycle, Ac-Phe-[Orn-Pro-D-Cha-Trp-Arg], a Potent New Anti-Inflammatory Drug *J. Org. Chem.* 68: 4464-71, 2003

91 Proctor, LM Arumugam, TV, Shiels, IA, Woodruff,TM, Reid, RC Fairlie, DP and SM Taylor Comparative Anti-inflammatory Activities of Antagonists to C3a and C5a Receptors in a Rat Model of Intestinal Ischaemia/Reperfusion Injury *Br J Pharmacol* Accepted for Publication 2004

92 Arumugam, TV IA Shiels, TM Woodruff, DN Granger and SM Taylor The role of the complement system in ischemia-reperfusion injury: a review *Shock* 21:401-409, 2004

93 Arumugam, TV , TM Woodruff, LM Proctor, S Pollitt, SZ Stocks, IA Shiels, RC Reid, DP Fairlie and SM Taylor. Protective effect of a human C5a receptor antagonist against hepatic ischaemia-reperfusion injury in rats *J. Hepatol.* In Press 2004

94 Arumugam TV, TM Woodruff, LM Proctor, KA Hansford, C Reid, IA Shiels, DP Fairlie and SM Taylor Beneficial effects of a new $sPLA_2$ inhibitor and C5a receptor antagonist against cold storage-induced ischaemic liver and renal injury in UW solution Submitted

95 March, DR LM Proctor, MJ Stoermer, R Sbaglia, G Abbenante, RC Reid, K Wadi, JD Tyndall, SM Taylor, and DP Fairlie. Potent cyclic antagonists of the complement C5a receptor on human polymorphonuclear leukocytes. Structure-activity relationships *Mol. Pharmacol.* 65:868-79, 2004

96 Woodruff, TM, N Dryburgh, RC. Reid, KA. Hansford, DP. Fairlie, SM.Taylor and IA.Shiels Anti-Inflammatory Properties of an Orally Active Secretory Phospholipase A_2 Inhibitor in Antigen-Induced Monoarticular Arthritis in the Rat. Submitted

97 Cain, SA, Taylor, SM, Proctor LM, Fairlie, DP and PN Monk The effects of mutating putative activation switch residues on the activity of a cyclic peptide C5a receptor antagonist Submitted

98 Shiels,IA, M Pappas, R Reid, DP Fairlie, SM Taylor. An inhibitor of the secretory phospholipase A_2 enzyme reduces contractions in rat uterus. Submitted

99 Proctor,LM, Woodruff,TM, Packowski,NJ, Strachan,AJ, Monk,PM, Reid,RC, Ross,PA, Fairlie DP and SM Taylor. Peptide Analogues of the C-terminus of C3a and C5a have Differential Hemodynamic Effects in the Rat Submitted.

100 Woodruff, TM, TV Arumugam, IA. Shiels, M L Newman, RC Reid, DP Fairlie, and SM Taylor. A Potent and Selective Inhibitor of Group IIa Secretory Phospholipase A_2 Protects Rats From TNBS-Induced Colitis submitted

Book Chapters

Taylor SM & DP Fairlie Discovery of Potent Cyclic Antagonists of Human C5a Receptors. In: Structural Biology of the Complement System. 2004 Eds. J Lambris and D Morikis Marcel Dekker NY USA.

Patents

1 Sanderson SD, Kirnarsky L, Sherman S, & SM Taylor. High affinity response-selective C-terminal analogs of C5a. *U.S. Patent 5,942,599* Issued 24/8/1999

2) *Taylor, S. M.; Fairlie, D. P. "Cyclic Agonists and Antagonists of C5a Receptors and G Protein-Coupled Receptors"* Aust. Patent 744991, 25 June 1997. International Patent PCT/AU98/00490, Filed 25 June 1998.

3) Shiels, I.; Taylor, S.M.; Fairlie, D. P. "A new method for the treatment of dysmenorrhoea and menstrual side effects : the use of inhibitors of human secretory phospholipases A2" filed as "Compositions and methods of using them" Aust. Provisional Patent, filed 14 July, 2000, PQ8764/00. *International Patent Application* 2004, PCT/AU01/00858

4) Taylor SM, Shiels IA and Brown LC. Use of C5a Receptor Antagonist in the Treatment of Fibrosis. *International Patent PCT/AU03/00415, Filed 8 April 2004.*

5) Taylor SM, Shiels IA, Fairlie DP. G Protein-Coupled. Receptor Antagonists. *International Patent PCT/AU02/01427, Filed 17 October 2002*

6) Woodruff TM and Taylor SM. Treatment of Inflammatory Bowel Disease. *International Patent PCT/AU2003/001365, Filed 17 October 2002.*

7) Taylor, S. M., Shiels, I.A Treatment of osteoarthritis by C5a antagonists. International Patent PCT/AU2003/001373, Filed 17 October 2002.

8) Shiels IA, Taylor SM and Fairlie D. Treatment of Hypersensitivity Conditions. *International Patent PCT/AU2003/001374, Filed 17 October 2002.*

9) Lindsay T, Harkin D and Taylor SM. Treatment of Haemorrhagic Shock. *Australian Patent Application No 2003902354, Filed 15 May 2003.*

10) Taylor SM and Shiels IA. Treatment of Burns. *Australian Patent Application No 2003902586, Filed 26 May 2003.*

11) Woodruff TM, Shiels IA and Taylor SM Treatment of neurological conditions. Australian Provisional Patent Application , *Filed April 2004.*

EXHIBIT 2

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-2

This is Exhibit SMT-2 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

A person empowered to witness Statutory
Declarations under the laws of the Queensland,
Commonwealth of Australia

ARTHRITIS & RHEUMATISM
Vol. 46, No. 9, September 2002, pp 2476–2485
DOI 10.1002/art.10449

Antiarthritic Activity of an Orally Active C5a Receptor Antagonist Against Antigen-Induced Monarticular Arthritis in the Rat

Trent M. Woodruff,[1] Anna J. Strachan,[1] Nathan Dryburgh,[1] Ian A. Shiels,[2] Robert C. Reid,[1] David P. Fairlie,[1] and Stephen M. Taylor[2]

Objective. **To determine if the new, orally active C5a receptor antagonist, the cyclic peptide AcF-[OPdChaWR], reduces the severity of pathology in a rat model of immune-mediated monarticular arthritis.**

Methods. **Arthritis was induced in the right knee of previously sensitized rats by the intraarticular injection of methylated bovine serum albumin. Rats were examined for either 14 days or 28 days, or for 49 days following a second antigen challenge at 28 days. The C5a antagonist (1 or 3 mg/kg/day) and/or ibuprofen (30 mg/kg/day) were administered orally on a daily basis either before or after arthritis induction.**

Results. **Rats receiving AcF-[OPdChaWR] had significant reductions in right knee swelling, gait disturbance, lavaged joint cell numbers, and right knee histopathology, as well as in serum levels of tumor necrosis factor α (TNFα) and intraarticular levels of interleukin-6 and TNFα on day 14. In the 14- and 28-day studies, ibuprofen resulted in a similar reduction in gait abnormalities and intraarticular inflammatory cells compared with the C5a antagonist, but was less effective in reducing knee swelling over the course of the study and had no effect on knee histopathology. Combination therapy with AcF-[OPdChaWR] and ibuprofen resulted in no greater efficacy than with the C5a antagonist alone. Rats injected twice with the antigen in the 49-day study displayed the most severe histopathology and this, as well as knee swelling and gait abnormalities, was significantly reduced by repeated treatment with the C5a antagonist.**

Conclusion. **An agent that inhibits the action of C5a in this model significantly reduced joint pathology, while ibuprofen was not effective. C5a antagonists could therefore have broader therapeutic benefits than nonsteroidal antiinflammatory drugs as antiarthritic agents for rheumatoid arthritis.**

Complement activation produces the 74-residue protein C5a, which is the most potent of the anaphylatoxins (1). C5a mediates numerous immune and inflammatory functions, including chemotaxis and activation of inflammatory cells, increased vascular permeability, spasmogenesis, immune regulation, and the release of a variety of inflammatory cytokines and mediators (1–3).

Rheumatoid arthritis (RA) is an immune complex disease involving the local activation of inflammatory cells, predominantly in the smaller peripheral joints. The complement system, and in particular the factor C5a, has long been identified as a likely contributor to the pathogenesis of RA. Elevated levels of C5a have been found in the plasma and inflamed joints of patients with RA (4,5). The number of C5a receptors on synovial mast cells is also increased in RA (6). Most important, the degree of complement activation in the joint and circulation correlates with the severity of the disease, suggesting that C5a plays a central role in disease pathogenesis (7–10).

Several animal models of RA have been used to elucidate the role of complement and C5a in this

Presented in part at the International Workshop on Complement Associated Diseases, Animal Models, and Therapeutics, Santorini, Greece, October 2001.

Supported in part by a grant from the National Health and Medical Research Council of Australia.

[1]Trent M. Woodruff, BSc, Anna J. Strachan, BSc, Nathan Dryburgh, BSc, Robert C. Reid, PhD, David P. Fairlie, PhD: University of Queensland, St. Lucia, Australia; [2]Ian A. Shiels, BVSc, PhD, Stephen M. Taylor, PhD: University of Queensland, St. Lucia, Australia/Promics Pty Ltd, St. Lucia, Australia.

Address correspondence and reprint requests to Stephen M. Taylor, PhD, School of Biomedical Sciences, Department of Physiology and Pharmacology, University of Queensland, St. Lucia, Queensland 4072, Australia. E-mail: s.taylor@mailbox.uq.edu.au.

Submitted for publication February 13, 2002; accepted in revised form April 25, 2002.

disease. Depletion of complement with cobra venom factor has been shown to delay the disease onset in a number of laboratory models of RA, including adjuvant arthritis, collagen-induced arthritis (CIA), and antigen-induced arthritis (AIA) in rats (11–13). Administration of recombinant soluble complement receptor 1 (sCR1) or anti-C5 antibodies, either intraperitoneally (IP), intravenously (IV), or intraarticularly (IA), has been shown to reduce disease progression in anti-CD59 antibody–induced models of acute arthritis (14), CIA (13,15), and AIA (12). However, the role of C5a as a pathogenic factor in these models has been difficult to prove due to the lack of availability of a specific C5a antagonist.

This issue was addressed recently with the use of a peptidic C5a receptor antagonist, MeFKPdChaWr, in a model of membrane attack complex (MAC)–dependent anti-CD59 antibody-induced arthritis (14). Administration of the antagonist, either IV or IA, failed to inhibit the development of disease parameters (14). Explanations for the apparent lack of C5a involvement in this animal model include the possibility of impaired clearance of MAC rather than activation of the complement cascade, failure of pathology to be affected by complement depletion, and resolution of the lesion within 3 days (16).

Current drug therapies for RA remain relatively ineffective at retarding disease progression and are associated with significant side effects (for review, see ref. 17). Drugs such as the nonsteroidal antiinflammatory drugs (NSAIDs), which include ibuprofen, provide palliative relief of symptoms, but have little beneficial effect on the pathology and progression of disease (17,18). Patients with developing RA require an effective, orally active therapy that will moderate the symptoms as well as arrest the progression of the destructive joint pathology.

Researchers in our laboratory have recently developed a series of small molecule antagonists of the human C5a receptor (19,20). One of these is a cyclic peptide, AcF-[OPdChaWR], which is orally active and has been shown to effectively reduce the severity of diseases in various rat models (21–23). In the present study, we tried to use this specific C5a receptor antagonist to determine the relative contribution of C5a in the pathogenesis of immune-mediated monarticular arthritis and assess the effectiveness of the antagonist in treating the disease symptoms and pathology. This compound has been shown to be a potent antagonist of both human and rat C5a receptors on polymorphonuclear leukocytes (PMNs) (24). It is also a potent antagonist of C5a receptors on human macrophages (25). We therefore hypothesized that any demonstration of antiarthritic activity in rats would bode well for future testing in human arthritic conditions. The data herein demonstrate that oral administration of AcF-[OPdChaWR], either before or after induction of the disease, is effective at preventing the development of pathology and moderating disease progression. These findings confirm the importance of C5a in this disease model and suggest a future role for antagonists of the C5a receptor in the treatment of immune-mediated arthritis and other inflammatory diseases.

MATERIALS AND METHODS

Animals. Female Wistar rats weighing 225–275 gm were used in this study. All animal experimentation conducted in this study was performed in accordance with National Health and Medical Research Council of Australia guidelines.

Antagonist preparation. The cyclic peptide, AcF-[OPdChaWR] (AcPhe[Orn-Pro-D-cyclohexylalanine-Trp-Arg]), was manufactured as previously described (20). The compound was purified by reverse-phase high-performance liquid chromatography and fully characterized by mass spectrometry and proton nuclear magnetic resonance spectroscopy.

Experimental procedure. Rats were sensitized by subcutaneous injection of 0.5 mg methylated bovine serum albumin (mBSA; Sigma, St. Louis, MO) in 0.5 ml Freund's complete adjuvant (Sigma) on day 21 and day 14 prior to challenge. Two weeks after the second injection (day 0), rats were anesthetized with ketamine (80 mg/kg IP, ilium; Lyppard, Brisbane, Australia) and xylazine (12 mg/kg IP, ilium; Lyppard) and both hind legs were shaved. A suspension of 0.5 mg mBSA in 100 μl saline was aseptically injected into the joint space of the right knee, with the contralateral left knee receiving saline alone. Swelling of the right and left knees was quantitatively assessed at various times during the study period by measuring the medial-lateral width across the joint with Vernier calipers. The appearance of each rat's gait was also scored (0–4) independently in a blinded manner during the study. A normal gait (no limp and full weight-bearing ability on the right hind leg) was scored as 0; a mild limp on the right hind leg was scored 1; a moderate limp on the right hind leg was scored 2; a severe limp on the right hind leg was scored 3; and no weight-bearing ability on the right hind leg was scored 4.

At the completion of each study, left and right knee joints were lavaged with 100 μl saline and total and differential cell counts were performed. The fluid was then centrifuged (11,000g, 3 minutes) and the supernatant stored at −20°C until analyzed for concentrations of tumor necrosis factor α (TNFα) and interleukin-6 (IL-6). A sample of blood was also obtained, allowed to clot, and centrifuged (11,000g, 3 minutes), with the resulting serum stored at −20°C for later determination of TNFα concentrations. The knee joints from every rat were dissected out, stored in 10% buffered formalin for at least 10 days, and decalcified in a saturated solution of EDTA for 21

days. They were then embedded in paraffin, and sections were cut, mounted, and stained using hematoxylin and eosin.

Study design and treatment groups. Three separate experimental trials were conducted lasting either 14, 28, or 49 days after arthritis induction. The trials lasting 14 or 28 days were performed twice and data were pooled for the final analysis. There were no differences in results between the replicate experiments. A very small proportion of sensitized rats (4 of 130) showed no knee swelling after intraarticular injection of antigen; these rats were excluded from the study.

In the initial experimental trial, rats were monitored for 14 days after arthritis induction. One group of rats received 1 mg/kg/day AcF-[OPdChaWR] in the drinking water, commencing 2 days prior to the induction of arthritis on day 0 and continuing throughout the study. The control group received water only. It was initially determined that rats drink 25 ± 3 ml water per day (mean ± SEM; n = 8), and drinking volumes were monitored daily during the experiments to validate the dose delivery. Administration of the C5a antagonist at this dose in the drinking water of rats for 7 days was effective at completely blocking C5a-induced neutropenia and the binding of C5a to neutrophils isolated from these rats (data not shown). The prolonged in vivo activity of the C5a antagonist following a single oral dose (22) also validated this dose regimen. Another group of rats received either AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (sodium salt, 30 mg/kg/day; Sigma) in the drinking water from day +2 onward, with control rats receiving water alone.

In the second study, rats were monitored for 28 days after arthritis induction. Animals received either AcF-[OPdChaWR] (1 mg/kg/day), ibuprofen (sodium salt, 30 mg/kg/day), or a combination of AcF-[OPdChaWR] (1 mg/kg/day) and ibuprofen (sodium salt, 30 mg/kg/day) in the drinking water from day −2 onward. Control animals received water only. A separate group of rats received AcF-[OPdChaWR] (3 mg/kg/day) in the drinking water from day +4 onward, with control rats receiving water only.

In the third study, rats were monitored for 49 days after induction of arthritis, with rats being reanesthetized on day 28 with a second injection of 0.5 mg mBSA in 100 μl saline in the right knee and saline alone in the left knee. In this study, one group of rats received 1 mg/kg/day AcF-[OPdChaWR] in the drinking water from day −2 and throughout the study, with the arthritic control rats receiving water only.

TNFα and IL-6 measurement. Samples of serum and intraarticular lavage fluid were assayed for TNFα levels using an enzyme-linked immunosorbent assay (ELISA) kit (PharMingen, San Diego, CA) with a 1:10 dilution of samples as previously described (21,22). Intraarticular levels of IL-6 in the lavage fluid were determined using an ELISA method as previously described (21,22). Concentrations of TNFα and IL-6 in the samples were determined by linear regression analysis from the standard curve.

Histologic evaluation. Histologic sections from the right and left stifle joints of each animal were examined by an independent observer (IAS) in a blinded manner and graded on a scale of 0–4. A grade of 0 involved no detectable abnormalities. Pathology of grade 1 had some inflammatory cell infiltration in the synovial membrane with no significant thickening of the membrane or cartilage erosion. Joints with extensive inflammatory cell infiltration and thickening of the synovial membrane were scored 2. Joints with a more severe lesion were scored as 3, while the most severe lesion (scored 4) showed significant thickening and fibrosis of the joint capsule, involvement of the articular cartilage, appearance of inflammatory cells in the joint space, and extensive synovitis.

Statistical analysis. All experimental results are expressed as the mean ± SEM. Data were analyzed using GraphPad Prism 3.02 software (GraphPad Software, San Diego, CA). Statistical comparisons were made between drug-free arthritic rats and all other treatment groups or between the arthritic right knees and saline-injected left knees, using either Student's *t*-test or the Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Fourteen-day study. Measurements of the saline-injected left knee of each rat did not significantly change from preinjection values during the course of each experiment (data not shown). Following the injection of mBSA on day 0 in drug-free sensitized rats, the average increase in the width of the right knee peaked on day +3 (4.69 ± 0.32 mm; n = 11) (Figure 1A). Rats that had been pretreated from day −2 with AcF-[OPdChaWR] (1 mg/kg/day) had significantly ($P < 0.05$) reduced right knee widths from days +2–14 (peak on day +3 2.08 ± 0.59 mm; n = 9) compared with drug-free arthritic rats (Figure 1A). Gait scores in drug-free arthritic rats also increased above baseline levels following induction of arthritis (Figure 1B). Pretreatment with the C5a antagonist significantly decreased these scores from days +2–14 ($P < 0.05$). There was a high correlation between gait scores and knee swelling in drug-free arthritic rats for all 3 experimental trials (14-day trial $r^2 = 0.83$, n = 7; 28-day trial $r^2 = 0.96$, n = 11; 49-day trial $r^2 = 0.84$, n = 17) from day +2 to completion of the study.

In a separate study, the effects of the C5a antagonist or ibuprofen on knee swelling and gait scores following the establishment of arthritis were examined. Rats treated with either AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (30 mg/kg/day) from days +2–14 had significantly reduced knee swelling (days +3–14) and gait scores (days +4–14) compared with arthritic rats that received no drug treatment ($P < 0.05$; Figures 1C and D).

In the initial 14-day study, TNFα and IL-6 levels were found to be elevated in the right knee lavage fluid on day 14, as were TNFα levels in the serum of drug-free arthritic rats (Figures 2A and B). Rats pretreated with the C5a antagonist had significantly lower levels of these cytokines ($P < 0.05$; Figures 2A and B) in the joint and serum on day 14. The majority (>90%) of cells recovered from the right knee lavage fluid on day 14 were

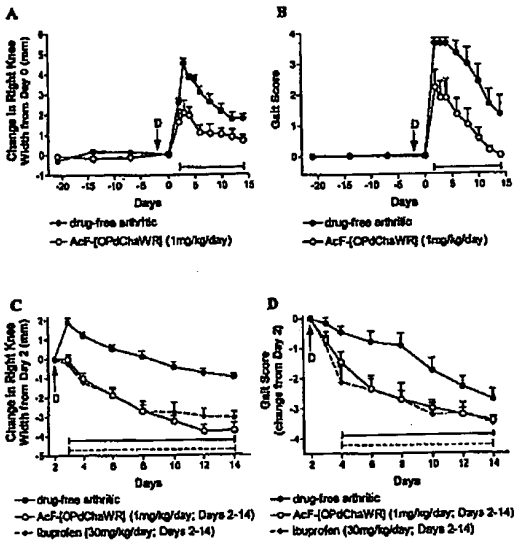


Figure 1. Right knee swelling and gait scores (14-day study). Antigen was injected into the right articular capsule of rats on day 0, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (30 mg/kg/day) from day +2 onward also had a significant reduction in knee swelling (C) and gait scores (D) compared with drug-free arthritic rats. Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars (solid for AcF-[OPdChaWR] and dashed for ibuprofen) ($P < 0.05$; n = 9–12). D = period when drug treatment began.

PMNs. Significantly fewer PMNs were found in the lavage fluid of rats treated with AcF-[OPdChaWR] or ibuprofen throughout the study, or in rats treated with the C5a antagonist from day +2, compared with drug-free arthritic rats ($P < 0.05$; Figure 2C).

The saline-injected left knees of all rats in every study showed no histologic abnormalities (Figure 3A) and all were scored 0 (data not shown). Sections from the right knees of drug-free arthritic rats on day 14 had marked cellular infiltration, which was predominantly neutrophils, and mild synovial cell proliferation, with a mean ± SEM histopathology score of 3.2 ± 0.3 (n = 16; Figures 2D and 3B and C). Histologic sections from rats pretreated with AcF-[OPdChaWR] had a lesser degree of cellular infiltration and synovial proliferation, resulting in a significantly lower histopathologic score of 1.4 ± 0.5 ($P < 0.05$; n = 8) (Figures 2D and 3D and E). Histologic sections from rats posttreated with the C5a antagonist 2 days after the induction of arthritis also had a lower degree of cellular infiltration and synovial proliferation compared with those from drug-free arthritic rats, although to a lesser extent than rats pretreated on day −2 with the C5a antagonist (Figure 3F).

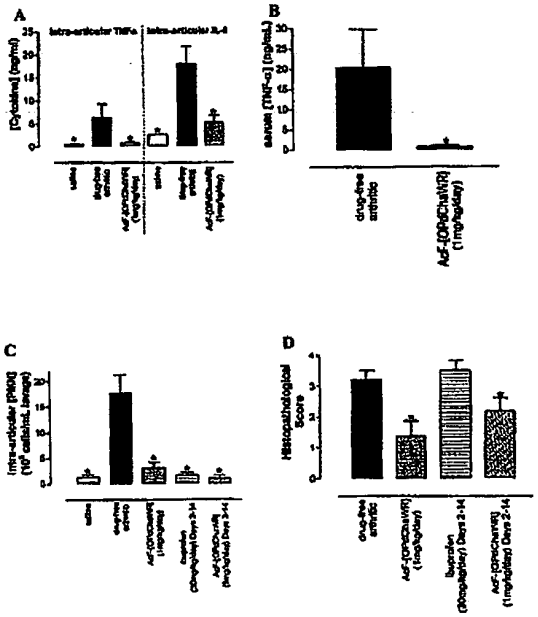


Figure 2. Intraarticular cytokine and polymorphonuclear leukocyte (PMN) levels, serum tumor necrosis factor α (TNFα) levels, and histopathologic scores (14-day study). Antigen was injected into the right articular capsule and saline into the left articular capsule of rats on day 0. On day 14, the animals were killed and the knee capsules lavaged for determination of TNFα and interleukin-6 (IL-6) levels, and PMN numbers. Serum was also analyzed for TNFα concentrations, and histopathologic analysis (scored 0–4) was performed on knee sections. A, Intraarticular TNFα and IL-6 concentrations were found to be significantly reduced in the right knee capsules of rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–14) compared with drug-free arthritic rats (n = 4–8). B, Serum levels of TNFα were also significantly reduced in rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–14) compared with drug-free arthritic rats (n = 8). C, Numbers of PMNs in the right knee intraarticular lavage fluid were significantly reduced in all drug treatment groups compared with right knee articular fluid from the drug-free arthritic rats (n = 8–12). D, Histopathologic scores in rats treated with AcF-[OPdChaWR] (1 mg/kg/day), either from days −2–14 or from days +2–14, were significantly lower than those in drug-free arthritic rats (n = 8–16). Rats treated with ibuprofen (30 mg/kg/day, days +2–14) had no improvement in histopathologic scores (n = 5). Data are expressed as the mean and SEM. * = $P < 0.05$.

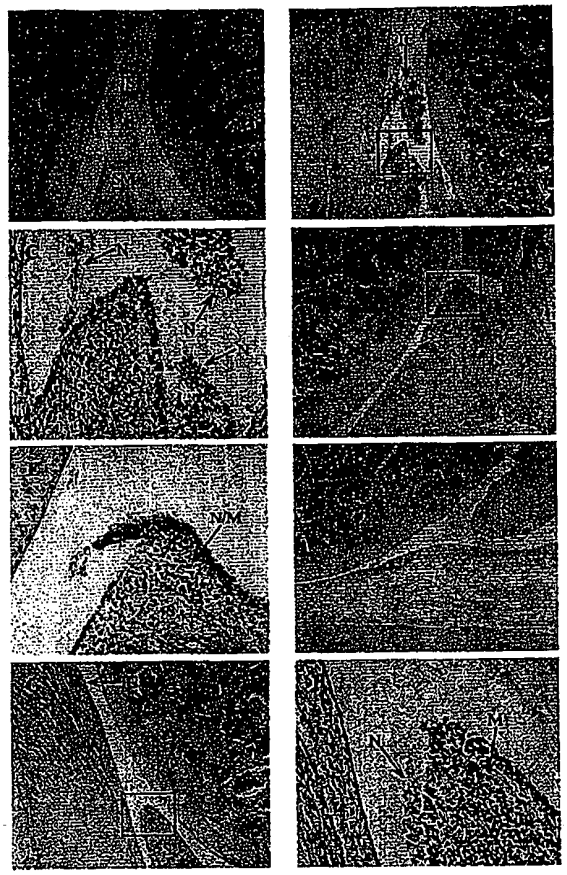


Figure 3. Sections of knees from rats in the 14-day study. A, Saline-injected left knee with normal synovial membrane (SM) and joint space (JS) (score 0). B, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) evident in the joint space (score 3). C, Higher magnification of the boxed area in B, with neutrophils (N) indicated. D, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–14)–treated rat (score 1). E, Higher magnification of the boxed area in D, with neutrophils and macrophages (M) shown. F, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days +2–14)–treated rat (score 1). G, Right knee of an ibuprofen (30 mg/kg/day, days +2–14)–treated rat (score 2.5). H, Higher magnification of the boxed area in G, with neutrophils and macrophages indicated. S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A, B, D, F, and G; ×200 in C, E, and H.)

The mean histopathologic score in these posttreated rats was 2.2 ± 0.4 (n = 8), which was significantly lower than that in drug-free arthritic rats ($P < 0.05$; Figure 2D). In contrast, histologic sections from rats treated with ibuprofen (30 mg/kg/day) from days +2–14 had no change in parameters (score 3.5 ± 0.3; n = 6) compared with those from drug-free arthritic rats (Figures 2D and 3G and H).

Twenty-eight–day study. Following arthritis induction, right knee widths of drug-free arthritic rats rapidly increased above baseline to peak on day +3 (4.98 ± 0.39 mm above baseline; n = 14) and slowly decreased to 0.51 ± 0.21 mm on day 28 (Figure 4A). Rats pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 had significantly lower right knee widths

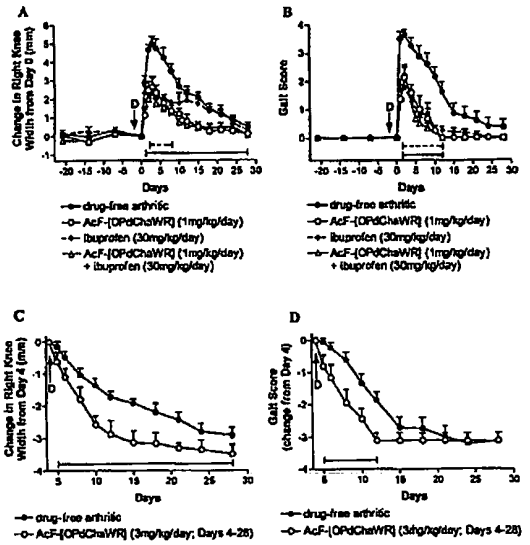


Figure 4. Right knee swelling and gait scores (28-day study). Antigen was injected into the right articular capsule of rats on day 0, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. Rats treated with ibuprofen (30 mg/kg/day, days −2–28) also had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats, but for a lesser time period than rats treated with the C5a antagonist for knee swelling. Rats treated with a combination of both the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) from day −2 onward also had significant reduction in these parameters (A and B), which was comparable with those in rats treated only with the C5a antagonist. Rats treated with AcF-[OPdChaWR] (3 mg/kg/day) from day +4 onward again had a significant reduction in knee swelling (C) and gait scores (D) compared with drug-free arthritic rats. Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars (dashed for ibuprofen and solid for AcF-[OPdChaWR] and AcF-[OPdChaWR]/ibuprofen combination) ($P < 0.05$; n = 11–16). D = period when drug treatment began.

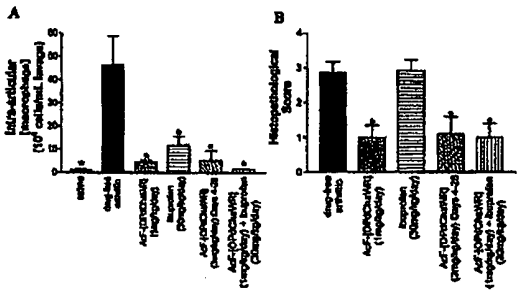


Figure 5. Intraarticular macrophages and histopathologic scores (28-day study). Antigen was injected into the right articular capsule and saline into the left articular capsule of rats on day 0. On day 28, the animals were killed, knee capsules were lavaged for determination of macrophage numbers, and histopathologic analysis (scored 0–4) was performed on stained knee sections. A, Macrophage numbers in the right knee intraarticular lavage fluid were significantly reduced in all drug treatment groups compared with right knee articular fluid from the drug-free arthritic rats (n = 6–10). B, Histopathologic scores from rats treated with AcF-[OPdChaWR] either from days −2–28 (1 mg/kg/day) or from days +4–28 (3 mg/kg/day), or in rats treated from days −2–28 with a combination of the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) were significantly lower than those from drug-free arthritic rats (n = 10–16). Rats treated with ibuprofen alone (30 mg/kg/day, days −2–28) had no improvement in histopathologic scores (n = 6). Data are expressed as the mean and SEM. * = $P <$ 0.05.

from days +1–28 (peak on day +3 2.66 ± 0.52 mm; n = 12) ($P < 0.05$; Figure 4A). Rats pretreated with ibuprofen (30 mg/kg/day) had significantly decreased knee widths from days +2–8 only (peak on day +3 2.30 ± 0.30 mm; n = 11) ($P < 0.05$; Figure 4A). Rats receiving a combination of the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) from day −2 had a significant reduction in right knee widths from days +1–28 (peak on day +3 2.19 ± 0.06 mm, n = 12) ($P < 0.05$; Figure 4A), which was comparable with that of rats receiving the C5a antagonist alone. Gait scores in rats pretreated with either the C5a antagonist, ibuprofen, or the combination of both were significantly reduced compared with those of drug-free arthritic rats from days +1–12 ($P < 0.05$; Figure 4B).

In the 14-day studies, which were performed first in the series reported here, the C5a antagonist was administered at 1 mg/kg/day both as a preventative (days −2–14) and as reversal (days +2–14) therapy. It was noted that rats treated with the antagonist at this dose from day +2 had less improvement in histopathologic scores compared with rats treated from day −2 (Figure 2D). In the following 28-day reversal studies, a higher dose of C5a antagonist (3 mg/kg/day) was used to determine if this might be a more effective reversal dose regimen. It was found that rats treated with this dose regimen had significantly improved right knee widths (days +5–28) and gait scores (days +5–12) compared with drug-free arthritic rats ($P < 0.05$; Figures 4C and D).

In all rats in the 28-day studies, there were no detectable levels of TNFα, either in the serum or in the knee lavage fluid, on day 28. The vast majority (>95%) of cells recovered in the lavage fluid of the right knees of rats were macrophages. Drug-free arthritic rats had an average of 46.5 ± 12.3 × 10^4 macrophages/ml lavage fluid (n = 10; Figure 5A), an ~20-fold increase over saline-injected left knees. Rats pretreated with AcF-[OPdChaWR], ibuprofen, or a combination of both, or rats treated from day +4 with the C5a antagonist alone, had significantly lower numbers of macrophages in the right knee lavage fluid (Figure 5A).

Histologic analysis of the right knees of arthritic drug-free rats revealed varying degrees of synovial cell proliferation and cellular infiltration, and these were scored overall, with an average of 2.9 ± 0.3 (n = 16; Figures 5B and 6A and B). Sections from rats either pretreated with C5a antagonist (1 mg/kg/day) or treated from day +4 (3 mg/kg/day) had an equal reduction in severity of lesions compared with drug-free arthritic rats, with significantly decreased scores of 1.0 ± 0.4 and 1.1 ± 0.5, respectively (n = 12 in each group) ($P < 0.05$; Figures 5B and 6C and H). Conversely, rats pretreated with ibuprofen (30 mg/kg/day) had no improvement in right knee pathology, with an average score of 2.9 ± 0.3 (n = 6; Figures 5B and 6D and E). Rats pretreated with a combination of the C5a antagonist and ibuprofen also had a significant reduction in histopathologic scores, which was similar to that in rats treated with only the C5a antagonist (n = 12; Figures 5B and 6F and G).

Forty-nine–day study. In drug-free rats following the first injection of mBSA on day 0, the right knee increased in width, with a peak swelling above baseline of 5.27 ± 0.56 mm (n = 6) on day +3. Following the second injection of mBSA on day 28, the increase in width of the right knee was similar in magnitude to the first challenge, with a peak of 4.47 ± 0.60 mm above preinjection values 1 day after the second injection (Figure 7A). Rats pretreated with AcF-[OPdChaWR] at 1 mg/kg/day had significantly lower right knee widths compared with drug-free rats from days +1–49, with peaks of 2.87 ± 0.51 mm above baseline (day 3) and 2.64 ± 0.37 mm (day 29) for the first and second injections, respectively (n = 6) ($P < 0.05$; Figure 7A).

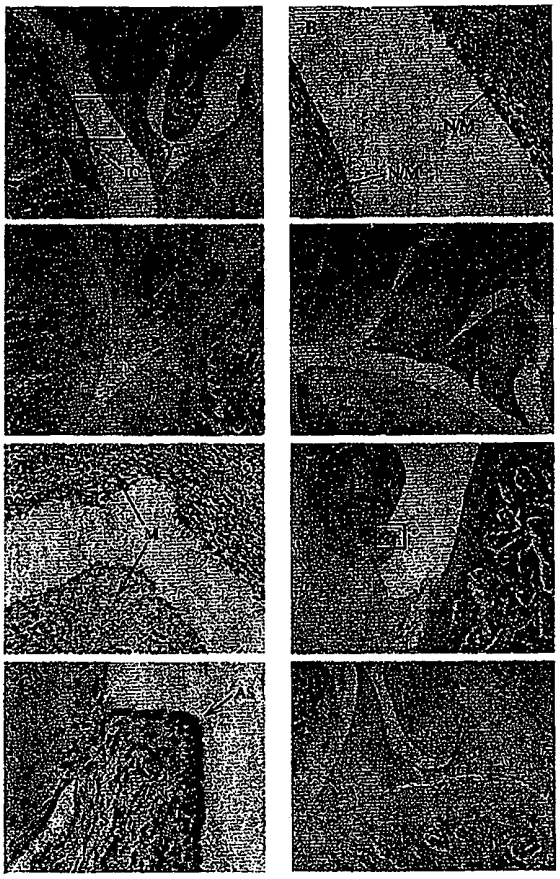


Figure 6. Sections of knees from rats in the 28-day study. A, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) lining the cartilage and areas of synovial fibrosis (F) indicated (score 2.5). B, Higher magnification of the boxed area in A, with neutrophils (N) and macrophages (M) evident. C, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–28)–treated rat (score 0); D, Right knee of an ibuprofen (30 mg/kg/day, days −2–28)–treated rat (score 3). E, Higher magnification of the boxed area in D, with macrophages shown. F, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day) + ibuprofen (30 mg/kg/day) combination (days −2–28)–treated rat, with the patella (P) indicated (score 1). G, Higher magnification of the boxed area in F, with activated synoviocytes (AS) shown. H, Right knee of an AcF-[OPdChaWR] (3 mg/kg/day, days +4–28)–treated rat (score 0). S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A, C, D, F, and H; ×200 in B, and E; × 400 in G.)

Gait scores increased in drug-free arthritic rats, with the deterioration in gait proportional to right knee widths, and maximum gait scores following both the first and the second injection (Figure 7B). C5a antagonist–dosed rats had significantly improved gait scores from days +2–16 and +30–44 ($P < 0.05$; Figure 7B).

Examination of right knee sections from 49-day arthritic drug-free rats showed a more severe pathology than was seen in the other studies involving single intraarticular injection of antigen and more limited experimental time spans. All antigen-injected right knees in drug-free rats had marked inflammatory cell infiltration and severe synovial proliferation and fibrosis (Figures 8A and B). Additionally, cartilage erosion was observed in all sections from challenged right knees, resulting in an average histopathologic score of 3.9 ± 0.1 (n = 6, Figures 7C and 8A and B). Sections of right knees from rats that had been pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had decreased pathology compared with that of drug-free arthritic rats, with a significantly improved score of 1.7 ± 0.7 (n = 6, Figures 7C and 8C and D).

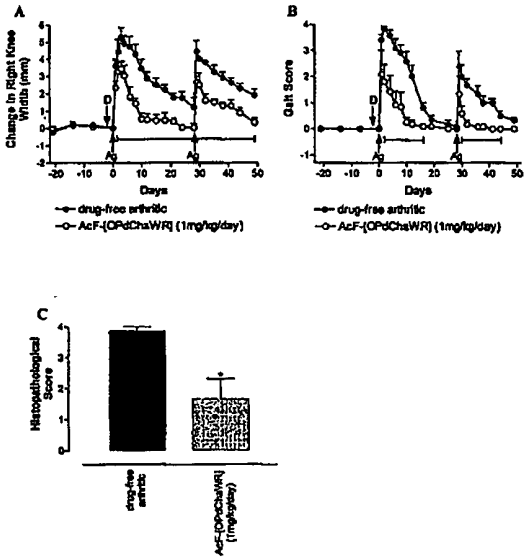


Figure 7. Right knee swelling, gait scores, and histopathologic scores (49-day study). Antigen was injected into the right articular capsule of rats on days 0 and 28, which resulted in increases in knee swelling and gait scores (scored 0–4). Rats treated with AcF-[OPdChaWR] (1 mg/kg/day) from day −2 onward had significantly reduced knee swelling (A) and gait scores (B) compared with drug-free arthritic rats. On day 49, animals were killed and histopathologic analysis (scored 0–4) was performed on stained knee sections. Rats treated with AcF-[OPdChaWR] (1 mg/kg/day, days −2–49) had significantly lower scores compared with drug-free arthritic rats (C). Results are expressed as the mean and SEM, with periods of significant difference from drug-free arthritic rats denoted by bars. D = period when drug treatment began; Ag = days antigen was injected. * = $P < 0.05$; n = 6.

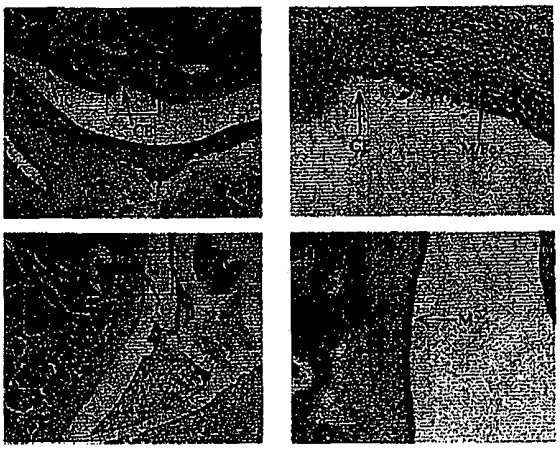


Figure 8. Sections of knees from rats in the 49-day study. A, Arthritic right knee of a drug-free rat, with inflammatory cells (IC) degrading the cartilage and a section of cartilage erosion (CE) shown (score 4). B, Higher magnification of the boxed area in A, with macrophages (M) and fibrocytes (Fc) shown alongside cartilage erosion. C, Right knee of an AcF-[OPdChaWR] (1 mg/kg/day, days −2–49)–treated rat, with mild appearance of inflammatory cells lining cartilage (score 1). D, Higher magnification of the boxed area in C, with macrophages shown. S = areas of synovitis. Images are typical and representative of each treatment group, and the score indicated is for the section shown. (Hematoxylin and eosin stained; original magnification ×40 in A and C; ×200 in B and D.)

DISCUSSION

Current drug treatment of RA involves the inhibition of cytokines and other mediators thought to be involved in its pathogenesis. The complement factor C5a is also recognized as a very important proinflammatory mediator in RA (6). Levels of C5a in the plasma and synovial fluid of patients with RA are higher than those found in patients with osteoarthritis (5). The pathology of RA includes the recruitment and accumulation of neutrophils and monocytes in the synovial tissues, with the down-regulation of C5a receptors in monocytes possibly contributing to chronicity of the disease (26,27). Synovial effusions contain high levels of C5a, which is a powerful chemoattractant for neutrophils and monocytes and invokes microvascular plasma leakage within the tissues (4,26). Inhibitors of activation of the complement system, or of the formation or action of C5a, have therefore been proposed to be of potential use in the treatment of RA. Anti-C5 monoclonal antibodies have been shown to block the development of CIA in rats as well as reduce the progression of established pathology (15). A recombinant protein inhibitor of complement activation, sCR1, has been suggested as a potential therapy for RA because efficacy with this compound has been shown in various animal models of arthritis (12,13,28).

The model of an antigen-induced monarticular Arthus reaction produces a discrete lesion of highly reproducible severity in a single joint, leaving the contralateral joint available for comparison. It has been suggested that the changes in the joint in clinical RA are due to this reaction (29). Neutrophils predominate in the synovial fluid in RA, particularly in the early stages, and this is mirrored in this rat model (30). Mononuclear cells were the predominant inflammatory cells in the rat synovium; this is also the case in RA (31). Additionally, the presence of the proinflammatory cytokines TNFα and IL-6 in the arthritic knee joint and TNFα in the serum of rats in the 14-day study correlates with the presence of these cytokines in the joints of arthritis patients (32).

The findings reported here show that an orally active, small molecule antagonist of the C5a receptor reduces the disease severity in a rat model of immune-mediated arthritis. Given on a daily basis, either before the initiation of arthritis or after symptoms were detectable, the drug reduced both joint swelling and gait symptoms. In the latter treatment case, drug therapy was started either 2 or 4 days after the lesion was stimulated in order to mimic the human clinical situation where a patient would present with an acute progressive lesion. In the 14-day study, PMNs were the predominant cell recovered in joint lavage fluid, whereas in the 28-day study, macrophages were the principal cell type found. In these shorter-term studies, cartilage erosion did not occur. In order to stimulate erosion, it was necessary to prolong the duration of the study and rechallenge the rats with antigen. Under these conditions, the C5a antagonist was again effective in reducing the degree of structural change in the joint.

These results show that this class of drug has multiple activities at different stages of the disease process. In previous short-term studies involving endotoxic shock and the Arthus reaction in the peritoneum and the dermis, the C5a antagonist was very effective at inhibiting the inflammatory challenges (21,22,33). The results of the present study demonstrate the efficacy of this class of drugs in a model of chronic immune complex–mediated inflammation, following daily oral administration of the drug.

The destruction of cartilage in osteoarthritis results from the IL-1–stimulated degradation of proteoglycans and inhibition of chondrocyte proteoglycan synthesis (18). NSAIDs protect the joint from swelling and cellular infiltration, but have little effect on disease

progression, while glucocorticoids normalize proteoglycan synthesis (17,18). The NSAID ibuprofen diminishes the responses in the rat as measured by joint swelling and disturbance of gait, and these findings equate well with the response to most NSAIDs in the clinic (17,34). Ibuprofen is less successful in reducing the structural pathology in the rat joint, and this is also similar to human clinical findings (17,34). In contrast, the C5a receptor antagonist used in this study significantly reduces the degree of structural pathology in the joint as well as other signs of the disease in the rats. This ability to moderate structural changes in the joint is a clear advantage over most of the NSAIDs.

The C5a antagonist used in the present study is orally active, with peak circulating plasma levels around 0.1–0.3 μM following a single oral dose of 3 mg/kg (22). The drug has a high affinity for human C5a receptors, and is an insurmountable antagonist active at low nanomolar concentrations (25). The drug was developed by structural analysis of the effector portion of C5a, and is a cyclic peptide that is resistant to metabolic degradation in the gut and plasma (20). In rats, a single oral dose of 10 mg/kg inhibits the neutropenia response to IV C5a for up to 24 hours (22), and oral treatment for 7 days at a dose of 1 mg/kg completely blocks the binding of C5a to circulating PMNs. In the present study, administration of 1 mg/kg/day was also an effective oral dose for reducing the expression of the arthritis symptoms and joint pathology. This C5a antagonist does not inhibit the formation of MAC at concentrations up to 100 μM (Strachan AJ: unpublished observations), indicating that, at least in the rat model of monarticular arthritis, inhibition of C5a alone is sufficient for effective reduction of symptoms and pathology. Similar results have been found in the same rat model of arthritis for the protein sCR1, which inhibits the formation of C3a, C5a, and MAC (12). This is consistent with our hypothesis that C5a is a major pathogenic mediator of disease pathology in this model. The precise role of MAC in antigen-induced monarticular arthritis, however, remains to be fully established.

In this study, combination drug therapy with ibuprofen and the C5a antagonist was found to be no more effective than therapy with the C5a antagonist alone. C5a causes the release of eicosanoids when administered in vivo to a variety of animal species, and the effects of C5a on blood pressure are blocked by cyclooxygenase inhibitors (35–37). The lack of additional efficacy on all parameters with the combination therapy may be due to the fact that the eicosanoid cascade is blocked early on by the C5a antagonist. The increased efficacy of the C5a antagonist over ibuprofen for joint pathology may be due to the additional inhibition of expression of proinflammatory cytokines, such as TNFα and IL-6. The importance of TNFα in the pathology of RA has been clearly demonstrated in a number of studies and has led to the development of a soluble TNFα receptor–based treatment (etanercept) in RA (38,39). It has previously been shown that inhibitors of C5a reduce the expression of TNFα and IL-6 in other disease models in vivo (21–23,40,41). The ability of the C5a antagonist to inhibit the expression of TNFα indicates an early role for C5a in the inflammatory cascade, and suggests a pivotal role for the therapeutic use of C5a antagonists in RA. The disease-modifying properties of the C5a antagonist in the present study may be due to its capacity to inhibit formation of both these cytokines and eicosanoids, rather than to the inhibition of eicosanoids alone.

In summary, this study demonstrates for the first time that a small molecule C5a receptor antagonist, given orally, prevents some principal signs of arthritis and significantly reduces the joint damage caused by an immune-mediated monarticular arthritis in rats. The disease-modifying effects of this antagonist suggest a potential use for C5a antagonists as antiarthritic agents in the clinic.

ACKNOWLEDGMENTS

We wish to thank Promics Pty Ltd. for supplying the C5a antagonist and Paul Addison for expert technical assistance in the histologic preparation of knee joint samples.

REFERENCES

1. Bitter-Suermann D. The anaphylatoxins. In: Rother K, Till GO, editors. The complement system. Berlin: Springer-Verlag; 1988. p. 367–84.
2. Yancey KB. Biological properties of human C5a: selected in vitro and in vivo studies. Clin Exp Immunol 1988;71:207–10.
3. Haynes DR, Harkin DG, Bignold LP, Hutchens MJ, Taylor SM, Fairlie DP. Inhibition of C5a-induced neutrophil chemotaxis and macrophage cytokine production in vitro by a new C5a receptor antagonist. Biochem Pharmacol 2000;60:729–33.
4. Jose PJ, Moss IK, Maini RN, Williams TJ. Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in the acute inflammatory phase. Ann Rheum Dis 1990;49:747–52.
5. Hogasen K, Mollnes TE, Harboe M, Gotze O, Hammer HB, Oppermann M. Terminal complement pathway activation and low lysis inhibitors in rheumatoid arthritis synovial fluid. J Rheumatol 1995;22:24–8.
6. Kiener HP, Baghestanian M, Dominkus M, Walchshofer S, Ghannadan M, Willheim M, et al. Expression of the C5a receptor (CD88) on synovial mast cells in patients with rheumatoid arthritis. Arthritis Rheum 1998;41:233–45.
7. Brodeur JP, Ruddy S, Schwartz LB, Moxley G. Synovial fluid

levels of complement SC5b-9 and fragment Bb are elevated in patients with rheumatoid arthritis. Arthritis Rheum 1991;34: 1531–7.
8. Makinde VA, Senaldi G, Jawad AS, Berry H, Vergani D. Reflection of disease activity in rheumatoid arthritis by indices of activation of the classical complement pathway. Ann Rheum Dis 1989;48:302–6.
9. Morgan BP, Daniels RH, Williams BD. Measurement of terminal complement complexes in rheumatoid arthritis. Clin Exp Immunol 1988;73:473–8.
10. Kemp PA, Spragg JH, Brown JC, Morgan BP, Gunn CA, Taylor PW. Immunohistochemical determination of complement activation in joint tissues of patients with rheumatoid arthritis and osteoarthritis using neoantigen-specific monoclonal antibodies. J Clin Lab Immunol 1992;37:147–62.
11. Kourounakis L, Nelson RA Jr, Kupusta MA. The effect of a cobra venom factor on complement and adjuvant-induced disease in rats. Arthritis Rheum 1973;16:71–6.
12. Goodfellow RM, Williams AS, Levin JL, Williams BD, Morgan BP. Local therapy with soluble complement receptor 1 (sCR1) suppresses inflammation in rat monoarticular arthritis. Clin Exp Immunol 1997;110:45–52.
13. Goodfellow RM, Williams AS, Levin JL, Williams BD, Morgan BP. Soluble complement receptor one (sCR1) inhibits the development and progression of rat collagen-induced arthritis. Clin Exp Immunol 2000;119:210–6.
14. Mizuno M, Nishikawa K, Morgan BP, Matsuo S. Comparison of the suppressive effects of soluble CR1 and C5a receptor antagonist in acute arthritis induced in rats by blocking of CD59. Clin Exp Immunol 2000;119:368–75.
15. Wang Y, Rollins SA, Madri JA, Matis LA. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. Proc Natl Acad Sci U S A 1995;92: 8955–9.
16. Mizuno M, Nishikawa K, Okada N, Matsuo S, Ito K, Okada H. Inhibition of a membrane complement regulatory protein by a monoclonal antibody induces acute lethal shock in rats primed with lipopolysaccharide. J Immunol 1999;162:5477–82.
17. Moreland LW, Russell AS, Paulus HE. Management of rheumatoid arthritis: the historical context. J Rheumatol 2001;28:1431–52.
18. Van den Berg WB. Impact of NSAID and steroids on cartilage destruction in murine antigen induced arthritis. J Rheumatol Suppl 1991;27:122–3.
19. Wong AK, Finch AM, Pierens GK, Craik DJ, Taylor SM, Fairlie DP. Small molecular probes for G-protein-coupled C5a receptors: conformationally constrained antagonists derived from the C terminus of the human plasma protein C5a. J Med Chem 1998; 41:3417–25.
20. Finch AM, Wong AK, Paczkowski NJ, Wadi SK, Craik DJ, Fairlie DP, et al. Low-molecular-weight peptidic and cyclic antagonists of the receptor for the complement factor C5a. J Med Chem 1999;42:1965–74.
21. Strachan AJ, Woodruff TM, Haaima G, Fairlie DP, Taylor SM. A new small molecule C5a receptor antagonist inhibits the reverse-passive Arthus reaction and endotoxic shock in rats. J Immunol 2000;164:6560–5.
22. Strachan AJ, Shiels IA, Reid RC, Fairlie DP, Taylor SM. Inhibition of immune-complex mediated dermal inflammation in rats following either oral or topical administration of a small molecule C5a receptor antagonist. Br J Pharmacol 2001;134:1778–86.
23. Arumugam TV, Shiels IA, Woodruff TM, Reid RC, Fairlie DP, Taylor SM. Protective effect of a new C5a receptor antagonist against ischemia-reperfusion injury in the rat small intestine. J Surg Res 2002;103:260–7.
24. Woodruff TM, Strachan AJ, Sanderson SD, Monk PN, Wong AK, Fairlie DP, et al. Species dependence for binding of small molecule agonist and antagonists to the C5a receptor on polymorphonuclear leukocytes. Inflammation 2001;25:171–7.
25. Paczkowski NJ, Finch AM, Whitmore JB, Short AJ, Wong AK, Monk PN, et al. Pharmacological characterization of antagonists of the C5a receptor. Br J Pharmacol 1999;128:1461–6.
26. Kitsis E, Weissmann G. The role of the neutrophil in rheumatoid arthritis. Clin Orthop 1991;63–72.
27. Katona IM, Ohura K, Allen JB, Wahl LM, Chenoweth DE, Wahl SM. Modulation of monocyte chemotactic function in inflammatory lesions: role of inflammatory mediators. J Immunol 1991;146: 708–14.
28. Rioux P. TP-10 (AVANT immunotherapeutics). Curr Opin Investig Drugs 2001;2:364–71.
29. Weissmann G, Korchak H. Rheumatoid arthritis: the role of neutrophil activation. Inflammation 1984;8 Suppl:S3–14.
30. Kowanko IC, Ferrante A. Interferon-γ increases human neutrophil-mediated cartilage proteoglycan degradation. Clin Exp Rheumatol 1992;10:123–9.
31. Kinne RW, Brauer R, Stuhlmuller B, Palombo-Kinne E, Burmester GR. Macrophages in rheumatoid arthritis. Arthritis Res 2000;2:189–202.
32. Manicourt DH, Poilvache P, van Egeren A, Devogelaer JP, Lenz ME, Thonar EJ. Synovial fluid levels of tumor necrosis factor α and oncostatin M correlate with levels of markers of the degradation of crosslinked collagen and cartilage aggrecan in rheumatoid arthritis but not in osteoarthritis. Arthritis Rheum 2000;43:281–8.
33. Short A, Wong AK, Finch AM, Haaima G, Shiels IA, Fairlie DP, et al. Effects of a new C5a receptor antagonist on C5a- and endotoxin-induced neutropenia in the rat. Br J Pharmacol 1999; 126:551–4.
34. Langenegger T, Michel BA. Drug treatment for rheumatoid arthritis. Clin Orthop 1999;22–30.
35. Lundberg C, Marceau F, Hugli TE. C5a-induced hemodynamic and hematologic changes in the rabbit: role of cyclooxygenase products and polymorphonuclear leukocytes. Am J Pathol 1987; 128:471–83.
36. Regal JF, Fraser DG. Recombinant human C5a-induced bronchoconstriction in the guinea-pig: a histamine independent mechanism. Pulm Pharmacol 1990;3:79–87.
37. Ito BR, Roth DM, Engler RL. Thromboxane A2 and peptidoleukotrienes contribute to the myocardial ischemia and contractile dysfunction in response to intracoronary infusion of complement C5a in pigs. Circ Res 1990;66:596–607.
38. Camussi G, Lupia E. The future role of anti-tumour necrosis factor (TNF) products in the treatment of rheumatoid arthritis. Drugs 1998;55:613–20.
39. Alldred A. Etanercept in rheumatoid arthritis. Expert Opin Pharmacother 2001;2:1137–48.
40. Hopken U, Mohr M, Struber A, Montz H, Burchardi H, Gotze O, et al. Inhibition of interleukin-6 synthesis in an animal model of septic shock by anti-C5a monoclonal antibodies. Eur J Immunol 1996;26:1103–9.
41. Mulligan MS, Schmid E, Beck-Schimmer B, Till GO, Friedl HP, Brauer RB, et al. Requirement and role of C5a in acute lung inflammatory injury in rats. J Clin Invest 1996;98:503–12.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-3

This is Exhibit SMT-3 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Joni Paso

No 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

Physiological Characterization of mBSA Antigen Induced Arthritis in the Rat. II. Joint Blood Flow, Glucose Metabolism, and Cell Proliferation

SVEN E. ANDERSSON, ALF JOHANSSON, KRISTINA LEXMÜLLER, and GUNILLA M. EKSTRÖM

ABSTRACT. Objective. Based on the hypothesis that blood flow in the inflamed joint is inadequate to maintain aerobic glycolysis, we sought to estimate the correlation between blood flow, glucose metabolism, and cellular proliferation rate in the arthritic joint.

Methods. Experiments were performed on rats with antigen induced arthritis (AIA). Regional blood flows (RBF) were measured with the microsphere technique, glucose metabolism by determination of [^{14}C]2-deoxy-D-glucose (2-DG) uptake, and the proliferative response as the incorporation of [^{3}H]-thymidine.

Results. In periarticular soft tissue of the arthritic knee the only significant change in the weight related RBF was an approximate 70% rise on Day 14 after arthritis onset. The RBF was lowest on Day 3 and the time course for the changes was inversely related to intensity of vascular inflammation. Weight related 2-DG uptake was more elevated than the RBF and peaked on Day 3. [^{3}H]-thymidine incorporation in the soft tissue was only markedly enhanced on Day 3. Neither 2-DG nor [^{3}H]-thymidine uptake was affected by treatment with methotrexate or indomethacin. In epiphyseal bone RBF was reduced on the first day of arthritis, but steadily increased thereafter.

Conclusion. In AIA an intense vascular leakiness negatively affects the synovial blood. There is a marked enhancement of glucose metabolism, but only a minor part of this increase seems to be induced by increased cellular proliferation. (J Rheumatol 1998;25:1778–84)

Key Indexing Words:
ACIDOSIS ARTHRITIS BLOOD FLOW
GLUCOSE METABOLISM PROLIFERATION

Clinical findings have reported that there is a local lactate acidosis in the joints of some patients with rheumatoid arthritis (RA). As lactate levels are reported to correlate to the intensity of the arthritis, this variable could be important for the understanding of the arthritis process[1,2]. The acidosis indicates an imbalance where local blood flow fails to meet the metabolic demand. In a complex interaction, both proliferative and vascular inflammatory responses in the joint can contribute to this imbalance; the ingrowth of pannus exceeds the pace of new formation of blood vessels, causing a fall in capillary density (for references see[3]). There is also widespread obliterative microangiopathy creating underperfused areas in the pannus[4]. Furthermore, increased vascular leakiness and subsequent formation of intraarticular effusions would elevate the joint pressure and thus, in turn, compress the synovial blood vessels[3,5]. This is particularly pronounced during joint motion when the intraarticular pressure is reported to rise to levels exceeding synovial capillary perfusion pressure[6].

As both pannus growth and inflammatory activation of the cells in the joint require energy, metabolic imbalance is further aggravated by increased metabolic demand. In patients with RA this can be measured as increased oxygen consumption[7] and glucose uptake[8].

Ischemia can perpetuate the arthritis in several ways; transient periods can induce the formation of oxygen radicals and reperfusion injury[3,6]. Oxygen radicals are also involved in the activation of the transcription factor nuclear factor kappa B (see[9]). Poor nutrition can induce production of heat shock proteins that can activate immunological mechanisms in the development of arthritis (see[10]). Hypoxia itself can also promote pannus growth both by enhancing cellular proliferation[11] and by providing a selection pressure for cells with diminished apoptotic potential, in particular cells with mutations of the *p53* gene[12]. Interestingly, such cells have been reported to be overexpressed in RA synovial tissue[13]. The relationships between these vascular, proliferative, and metabolic factors are, however, difficult to study in humans and little is known about how they are affected in animal models of arthritis.

In a previous study, we found that in collagen induced arthritis (CIA) in the rat vascular porosity was markedly enhanced in joints, but that the changes in blood flow were

From the Department of Pharmacology, Preclinical Research and Development, Astra Draco AB, Lund; and Department of Physiology and Medical Biophysics, University of Uppsala, Uppsala, Sweden.

S.E. Andersson, MD, PhD, Assistant Professor, Department of Pharmacology, Astra Draco; A. Johansson, MSc, Department of Physiology and Medical Biophysics, University of Uppsala; K. Lexmüller, BSc; G.M. Ekström, PhD, Astra Draco.

Address reprint requests to Dr. S.E. Andersson, Department of Pharmacology, Astra Draco AB, PO Box 34, S-22100 Lund, Sweden.

Submitted November 4, 1997 revision accepted March 31, 1998.

much smaller and non-persistent[14]; these findings concur with the hypothesis of local ischemia in the joints. We deemed the antigen induced arthritis (AIA) model to be more suitable for studies of joint pathophysiology because (1) it has a higher reproducibility than CIA; and (2) only one knee is inflamed in AIA, hence the other serves as an internal control. As in RA, AIA is characterized by 2 essential features; inflammation and formation of aggressive pannus that degrades cartilage and bone[15–17]. In our adjacent work we studied these phenomena simultaneously and found very marked inflammation measured as increased vascular porosity, and which precedes the pannus growth[18]. According to the above hypothesis, this would lead to reduced blood flow which may be insufficient to meet the metabolic demand and, hence, lead to anaerobic glycolysis. To better understand this process, we tried to quantitate both the changes in blood flow and glucose metabolism in the arthritic joint of AIA rats. Also, in a companion article[18] we measured pannus growth as weight increase of the periarticular soft tissue and the effect of methotrexate (MTX) and indomethacin treatment on this variable. In the present study we extend that investigation by assessing the mechanism of pannus formation by measuring the cellular proliferation rate. Further, we determined how this rate is related to glucose consumption and was affected by pharmacological manipulation.

MATERIALS AND METHODS

The methodology is described in our companion work[18]; briefly, experiments were performed on female Dark Agouti (DA) rats (150 g) obtained from Möllegaards Breeding Centre, Køge, Denmark. The experiments were approved by the Animal Ethics Committee of Lund, Sweden. Immunization was performed by intradermal injection with 1 mg methylated bovine serum albumin (mBSA) dissolved in 50 µl saline and emulsified in 50 µl Freund's complete adjuvant. Ten days later the rats were challenged with an intraarticular injection of 50 µg mBSA (1 µg/µl, dissolved in saline) into one knee. The contralateral knee served as a control and was injected with the same volume of saline. Experiments were performed on Days 1, 3, 7, and 14 after arthritis induction.

Anesthesia, general surgical procedure. The rats were anesthetized with a 1:1 mixture of fluanisone/fetanyl (Hypnorm®) and midazolam (Dormicum®), both diluted 1:1 with distilled water before mixing and given 0.25 ml/rat subcutaneously. They were tracheotomized, allowed to breathe freely, and placed on a servo-controlled heating pad to maintain body temperature at about 37°C. The right jugular vein was cannulated with polythene tubing for intravenous (iv) administration. One carotid artery was also cannulated and used for reference blood sampling.

Blood flow measurements. The regional blood flow (RBF) of periarticular tissue and of epiphyseal bone were measured using the labelled microsphere method[19–21]. In brief, radiolabelled microspheres are injected into the left ventricle of the heart. The spheres are distributed with the cardiac output and are trapped in the precapillary sphincters. During injection, an arterial blood sample is taken, and based on the relationship between blood weight and radioactivity in that sample and the radioactivity in the studied tissue, RBF can be calculated. In our experiments, one carotid artery was catheterized with polyethylene tubing, which was connected by a Y-connection to a blood pressure transducer and to a peristaltic pump (Alitea, Stockholm, Sweden) for withdrawing the reference blood sample. To prevent clotting all catheters were flushed with heparin (5000 IE/ml) before the experiment. An injection needle connected to a syringe by polyethylene tubing was introduced into the left ventricle of the heart by direct puncture and used for injection of microspheres. The spheres had a diameter of 15 µm and were labelled with ^{141}Ce and suspended in saline with 0.1% Tween 80. The spheres were injected for 30 s and a reference blood sample was withdrawn during the first 2 min from the start of injection. About 1.0×10^6 spheres were given. After the injection a blood sample was taken from the heart needle for determination of the acid base balance and hence localization of the needle, whereafter the animals were killed by overdose of pentobarbital sodium given iv. The anterior part of the periarticular knee tissue was dissected out as described[18]. The remaining part of the knee was regarded as mainly consisting of epiphyseal bone. Samples were weighed and the radioactivity in them and in the reference blood sample was determined by gamma spectrometry. The RBF was then calculated according to the formula:

$$RBF = Q_r \cdot CPM_t/CPM_r$$

where Q_r = reference blood flow (g/min), CPM_t = radioactivity in the tissue sample, and CPM_r = radioactivity in the reference blood sample. Since validity of the microsphere method is dependent on an even distribution of the spheres, the spheres were vigourously shaken in a vortex mixer immediately before injection. Possible distortion was ruled out by determining that blood flow to the kidneys was symmetrical and plausible.

Autoradiographic analysis of glucose uptake. Glucose metabolism was measured as [^{14}C]2-deoxy-D-glucose (2-DG) uptake. This method was originally developed for measuring glucose metabolism in the brain[22] but has also been used in arthritic joints[8]. In the present experiments 15 µCi of 2-DG (56 Ci/mol) was injected iv and allowed to circulate for 40 min, whereafter the rat was killed and rapidly frozen in –70°C alcohol. The frozen knees were sagittally sectioned according to the Ullberg technique[23]. The [^{14}C] content of the tissues was then determined by densitometric analysis of autoradiographs. The densities of the autoradiograms were calculated in 240 × 230 picture elements (pixels) and expressed as a percentage of the value for the corresponding area in the control knee. Densities were calculated for the periarticular soft tissue (anterior and posterior joint capsule/pannus and suprapatellar bursa) and for the epiphyseal bone. n=2 for each day of study.

Quantification of glucose uptake and thymidine incorporation. [^{14}C]2-DG (15 µCi) and ^3H-thymidine (1 µCi; 84 Ci/mmol) were injected iv simultaneously and allowed to circulate for 50 min, whereafter the animals were killed and rapidly frozen in liquid nitrogen. Arterial blood samples were taken 5 min after injection and immediately before the end of the experiment. After thawing, the soft tissue of the knee was dissected out as described above. To relate uptake levels to those of a rapidly proliferating tissue, a piece of small intestine was taken for comparison. All tissue and blood samples were then stored at about –18°C until analyzed. The samples were thawed and then combusted in a Packard 307 oxidizer equipped with Oximate 80 (Packard, Meriden, CT, USA), a robotic system for sample processing. The combusted samples were automatically dissolved in 15 ml Monophase-S (Packard) for [^3H] and 12 ml Permaflour-E (Packard) for [^{14}C]. Radioactivity was measured on Tri Carb Spectrometers (Packard). Quench correction was performed by external standard procedures.

In a separate series the modulating effect of MTX (0.3 mg/kg) and indomethacin (2 mg/kg) on these variables was investigated. The rats were treated with an intraperitoneal injection of either compound on the second day of arthritis and the above variable was remeasured the next day.

Substances. Indomethacin (Confortid®) was purchased from Dumex, (Copenhagen, Denmark); methotrexate from Lederle (Wayne, NJ, USA); mBSA, Freund's complete adjuvant from Sigma Chemical Co. (St. Louis, MO, USA); Dormicum® from Roche (Basel, Switzerland); Hypnorm® from Jansen (Beerse, Belgium); microspheres from DuPont NEN (Wilmington, DE, USA); and [^3H]-thymidine and [^{14}C]2-DG from American Radiolabelled Chemicals Inc. (St. Louis, MO, USA).

Data, statistical calculations. All data are, unless stated otherwise, expressed as mean ± SEM and are the difference between the value measured in the control and the arthritic knee in the same rat. Statistical significance was calculated using analysis of variance, followed by the Fisher protected least significant difference or, if only 2 groups were compared, by Student's t test. All calculations were performed on an Apple computer using StatView 4.0 (Berkeley, CA, USA) software.

RESULTS

The intraarticular injection of mBSA induced arthritis in all challenged knees, measured as an increase in knee diameter.

The procedure using direct heart puncture for administration of microspheres sometimes causes cardiovascular disturbances that may affect cardiac output. This would likely affect the RBF in both knees alike. We therefore chose to relate the blood flows in the arthritic knees to those in the contralateral control knee. Values are thus expressed as percentage of the weight related (g/min/g) RBF in the control tissue. This also allowed comparisons between studies of changes in blood flow, glucose, and thymidine uptake and weight gain of the pannus. In the periarticular soft tissue, the RBF was significantly elevated only on Day 14. The time course for the changes showed that the mean blood flow of the arthritic knee was lowest on Day 3 of arthritis and then slightly lower in the arthritic knee than in the control (Figure 1A). In the epiphyseal bone, the RBF was decreased in the arthritic knee on Day 1 of arthritis but then increased steadily and was significantly elevated on Days 7 and 14 (Figure 1B).

In the autoradiographs of 2-DG uptake the average densities calculated per area unit was 60–171% higher in the soft tissue of the arthritic knee than in the contralateral control (Figure 2). Due to the limited number of animals, no conclusion could be drawn on the differences in uptake between different days after challenge. The uptake was unevenly distributed, with marked accentuation in restricted areas adjacent to the cartilage of the femoral head and around the suprapatellar bursa. For the epiphyseal bone the average density/area unit was 12–18% higher in the arthritic joint.

When the total uptake of 2-DG was determined by scintillation technique, the levels were elevated about 100–150% on all days, with a slight peak on Day 3. These results are thus in accordance with those obtained by autoradiography. For [^{3}H]-thymidine, there was a slight increase in uptake on the first day of arthritis and a marked elevation on Day 3, but the levels did not significantly differ from the control knee on Days 7 and 14 (Figure 3). There was only a weak correlation between thymidine and glucose uptake (0.49, $p = 0.01$ in arthritic knee; 0.47, $p = 0.015$ in the control). A disturbing factor that may have influenced our results was the plasma levels of 2-DG versus [^{3}H]-thymidine: compared to the 5 min levels, between 30 and 40% of [^{3}H]-thymidine and 40–50% of 2-DG remained at 50 min. Since the relative water content, and hence possibly the distribution volume, was increased in the periarticular soft tissue of the arthritic knee, this could explain part of the increase in tissue levels. However, since the blood concentration (dpm/mg) of both [^{3}H]-thymidine and 2-DG was usually lower than the tissue concentration in the small intestine and the arthritic knee, we believe that the contribution of the circulating tracer levels to the results is minor. For instance, on Day 3 the mean concentrations of 2-DG in the small intestine and in the arthritic knee were 43 ± 12%,

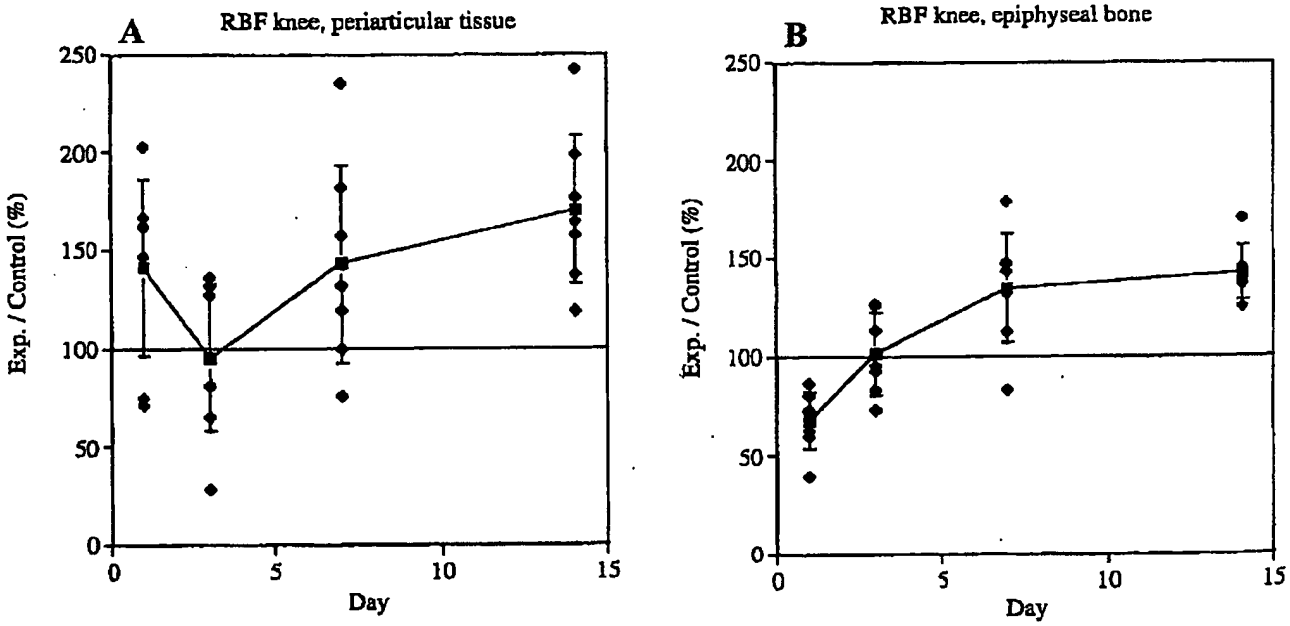


Figure 1. The weights related RBF (arthritis/control, %) in periarticular soft tissue (1A) and epiphyseal bone (1B). Results are given both as individual values and as means (95% confidence intervals).

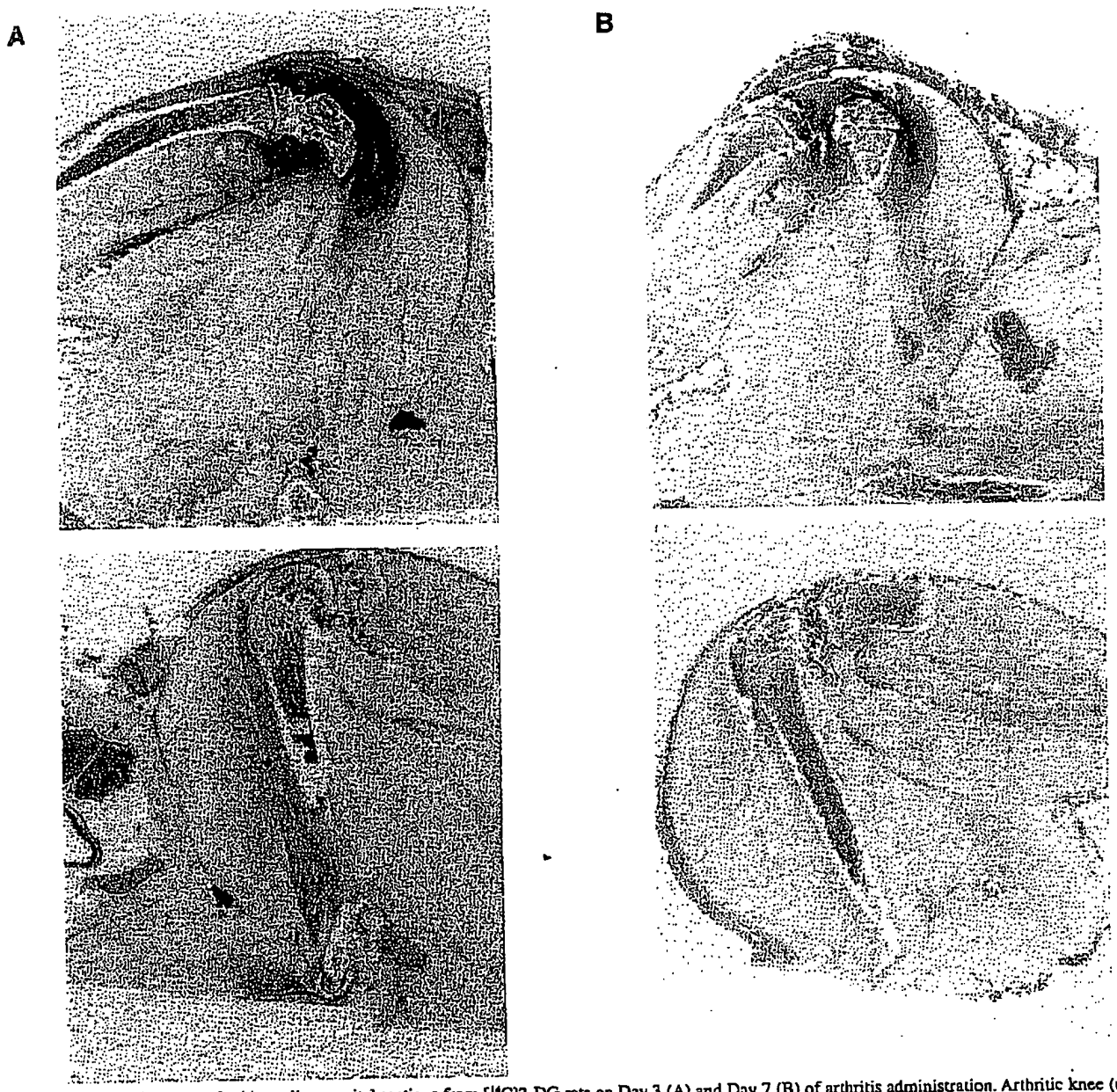


Figure 2. Autoradiographs of mid-patellar saggital sections from [^{14}C]2-DG rats on Day 3 (A) and Day 7 (B) of arthritis administration. Arthritic knee (upper panels) and contralateral control (lower panels). Note intense uptake in areas close to cartilage and around the suprapatellar bursa, which is distended on Day 3 (upper panel A).

61 ± 9% higher than in the blood, respectively. For [^{3}H]-thymidine the corresponding values were 844 ± 94% and 332 ± 12%, respectively.

When the ratio for weight related [^{3}H]-thymidine incorporation was plotted against the corresponding values for the pannus dry weight obtained in our adjacent paper[18], the observed increase was parallel between Days 1 and 3 but then differed markedly on the last 2 days of study (Figure 4). Both 2-DG uptake and [^{3}H]-thymidine incorporation were higher in the small intestine than in the control knee. In the arthritic joint, however, the 2-DG uptake was at about the same level as in the gut, whereas the [^{3}H]-thymidine incorporation was markedly lower. For example, on Day 3 of arthritis the values for 2-DG and [^{3}H]-thymidine content were 308 ± 6 and 40 ± 3 dpm/mg in the pannus tissue, respectively. Corresponding values for the small intestine were 278 ± 33 and 98 ± 14 and for the periarticular soft tissue of the control knee 124 ± 10 and 18 ± 1, respectively. The ratio between 2-DG and [^{3}H]-thymidine content was calculated to give an estimate of the amount of glucose

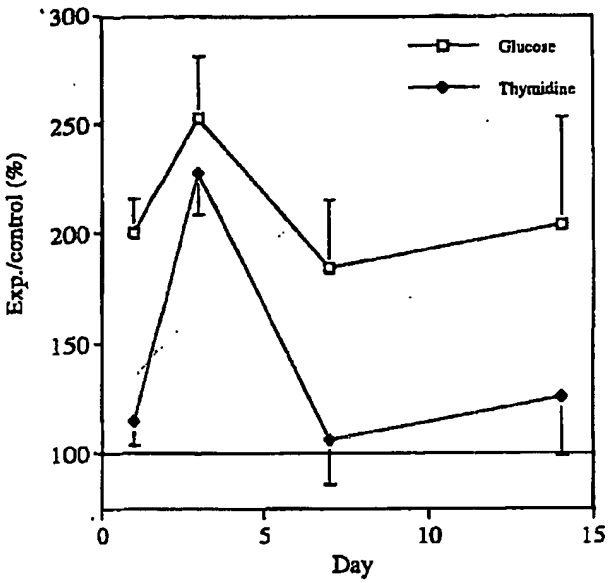


Figure 3. Weight related uptake (arthritis/control %; mean, with 95% confidence intervals) of [^{14}C]2-DG and [^{3}H]-thymidine in the periarticular soft tissue (n = 7 for Days 1, 3 and 7; Day 14 n = 5).

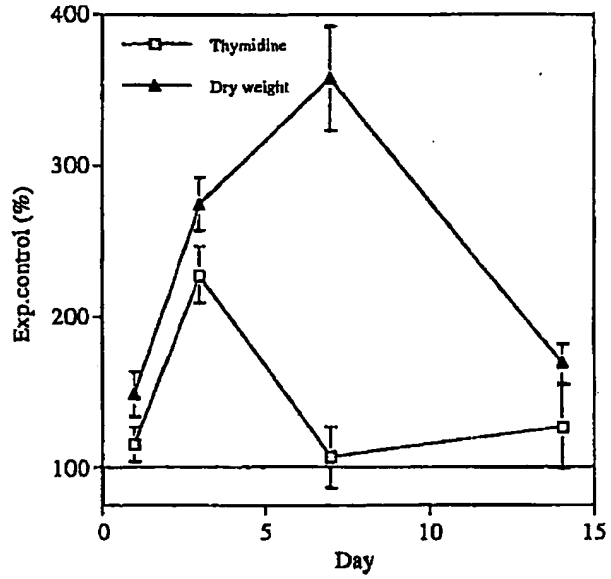


Figure 4. [^{3}H]-Thymidine uptake (dpm/mg) in the periarticular soft tissue compared with the increase in pannus dry weight from our companion paper[18] (both: arthritis/control %; mean, with 95% confidence intervals).

metabolism used for cell proliferation. This ratio was always lower in the small intestine than in the soft tissue of either knee. Comparing the knees, the ratio was significantly elevated in the arthritic knee on Days 1 and 7 of arthritis (Table 1).

As in our companion paper[18], indomethacin administered at Day 2 of arthritis reduced the vascular inflammation and pannus growth measured the next day, whereas the effect of

Table 1. Ratio for [^{14}C] 2-DG and [^{3}H]-thymidine content ([^{14}C]/[^{3}H]; both dpm/mg) in periarticular soft tissue of arthritic and control knees and small intestine on different days after arthritis induction. The ratio for the small intestine was the lowest in all experiments.

Day	Arthritic	Control (p)	Small Intestine
1	16.1 ± 2.7	9.3 ± 1.7 (0.013)	3.4 ± 0.3
3	8.0 ± 1.0	7.2 ± 1.0 (NS)	3.1 ± 0.4
7	15.2 ± 0.9	8.8 ± 0.9 (< 0.0001)	3.9 ± 0.5
14	17.3 ± 4.9	14.0 ± 6.8 (NS)	4.5 ± 1.3

Statistical significance was calculated between arthritic and control tissue using Student's 2 tailed t test for paired data. Periarticular tissue: n = 7 for Days 1–7; n = 5 for Day 14. Small intestine: n = 4–5.

MTX was smaller. Similar results were found in the present study: the difference in joint soft tissue weight between the knees was 232 ± 22 mg in the saline treated control group (n = 7), 172 ± 25 in the MTX group (n = 6, NS), and 156 ± 17 (n = 7) ($p < 0.05$ compared to control) in the indomethacin group. However, neither of the compounds affected the 2-DG uptake nor the [^{3}H]-thymidine incorporation. The ratios for the weight related uptake for 2-DG were: 308 ± 6%, 299 ± 12%, and 330 ± 14% for the control, MTX, and indomethacin groups, respectively. For [^{3}H]-thymidine, the corresponding values were 242 ± 37%, 224 ± 20%, and 242 ± 28%, respectively. These values are in good agreement with those found in the former series (Figure 4).

DISCUSSION

Blood flow; relation to intensity of vascular inflammatory response. The clinical response in AIA is a marked swelling of the arthritic knee, which peaks around Day 7 after challenge and is prominent for many weeks. Two variables may contribute to this measure: pannus growth and increased vascular porosity. In our companion paper[18], we found that the vascular inflammatory response, measured as leakiness for albumin, preceded the pannus growth and peaked around Day 3. In the present study, the RBF in the perivascular soft tissue of the arthritic knee was significantly elevated, compared to the control, only on Day 14. The time course for the changes was interesting: blood flow tended to increase on Day 1, drop to the lowest value on Day 3, and continued to increase thereafter in an inverse relationship with changes in vascular porosity. Hence, our findings suggest that, in this model, high vascular leakiness induces increased intraarticular pressure which, in turn, compresses synovial blood vessels; in other words, vascular inflammation negatively affects joint blood flow.

Glucose metabolism; localization and relation to blood flow. In contrast to RBF, 2-DG uptake was elevated more than 100% on all days studied. This discrepancy was especially marked on Day 3 when the mean RBF ratio was lowest and the 2-DG uptake was highest. This is compatible with the hypothesis that at that time, the oxygen supply would

not match glucose consumption and hence possibly lead to an ischemic situation. Conversely, ischemia may explain part of the increased 2-DG uptake since the energy yield per unit of glucose is much lower in anaerobic than in aerobic glycolysis. However, the present results do not allow conclusions regarding total energy metabolism, since we have no data on the consumption of triglycerides.

In addition to anaerobic glycolysis, it is possible that the 2-DG uptake is a marker of inflammatory activity. The autoradiographs indicated uneven distribution of 2-DG uptake, with "hot spots" in restricted areas close to the cartilage and around the suprapatellar bursa. Cationic antigens such as mBSA are deposited in the hyaline cartilage([24] and references) and it seems reasonable that the main foci of the inflammation lies adjacent to this antigen reservoir. In addition to the intense 2-DG uptake, autoradiographs revealed that the suprapatellar bursa was distended, and in a preliminary study using magnetic resonance imaging, marked plasma leakage seemed to occur in this area[25]. In our companion report[18] we also found a marked enhancement of cellular density in this area, which may explain the inflammatory activity and the augmented glucose uptake.

Relation between cellular proliferation rate and pannus growth. Local proliferation of fibroblast-like synovial cells has been observed in human rheumatoid synovia, but there is controversy about whether hyperplasia seen in the human disease is mainly caused by local proliferation or by influx of inflammatory cells[26]. In the present study the ratios (arthritis/control) for [^{3}H]-thymidine incorporation increased in tandem with the corresponding values for the dry weight of the soft tissue on the first and third days of arthritis, which proved to be the most intense phase of growth, before returning to baseline levels. This suggests that a substantial part of the initial pannus formation was due to cell proliferation, but that after a few days the increase in pannus weight may be more dependent on cellular influx or to increases in cell size. In our companion paper, we found that the pannus formation followed the inflammation in time and that it was probably induced by the latter. Since hypoxia is reported to enhance fibroblast proliferation[11] and induce release of growth factors such as vascular endothelial growth factor[27], it could be speculated that the presence of ischemia in the arthritic joint is an important link between inflammation and pannus formation.

Relation between cellular proliferation rate and glucose consumption. Effects of indomethacin and methotrexate. Cellular proliferation is an energy requiring process that, in addition to inflammatory activation and possible anaerobic glycolysis, may influence 2-DG uptake. We sought to obtain, by simultaneous co-administration of 2-DG and [^{3}H]-thymidine, a relative estimate of the amount of glucose used for cell proliferation. Among the compared tissues (the small intestine and the periarticular tissue in arthritic and control knees), the ratio between 2-DG and [^{3}H]-thymidine uptake was lowest, and the level of [^{3}H]-thymidine incorporation highest in the intestine, which is compatible with a high level of cell proliferation and a relatively large portion of the glucose being utilized for energy requiring processes associated with this. In the control knee the cell proliferation was low and the higher [^{14}C]/[^{3}H] ratio indicates that relatively more of the glucose was used in other processes such as basal metabolism. In the arthritic knee the 2-DG uptake was higher than in the control. A number of factors indicate that the extra glucose was not mainly used for cell proliferation. First, as mentioned, there was no marked enhancement of [^{3}H]-thymidine incorporation except on Day 3 and the [^{14}C]/[^{3}H] ratio was accordingly elevated compared to the control knee on Days 1 and 7. Second, on Day 3, when there was a peak in [^{3}H]-thymidine incorporation, the corresponding change in glucose metabolism was smaller and hence the [^{14}C]/[^{3}H] ratio returned to normal. Thus a marked increase in thymidine incorporation only affects the glucose metabolism to a minor extent. Third, there was only a weak correlation between 2-DG and [^{3}H]-thymidine levels in the periarticular soft tissue. This finding is in agreement with *in vitro* studies in which interleukin 1ß enhances glucose uptake by synoviocytes without enhancing cell proliferation[28]. The expression of this cytokine is elevated in murine AIA[29]; thus it seems likely that a large portion of the enhanced 2-DG uptake may be explained by the action of proinflammatory mediators that initiate energy requiring processes in the cells. However, there does not seem to be any clear coupling between glucose metabolism and vascular inflammation; on Day 14 there was still an increased metabolic activity in the joint measured both as 2-DG uptake and RBF, both in the soft tissue and the epiphyseal bone. This was not accompanied by significant inflammatory activity (measured as vascular leakiness) and the [^{3}H]-thymidine incorporation in soft tissue was not significantly elevated. In accordance, the reduction of vascular porosity by indomethacin on the third day of arthritis did not affect either glucose metabolism or cellular proliferation rate. Taken together, it seems that the glucose is mainly used for processes upstream of vascular inflammation but not directly correlated to this. It remains possible that some of the inflammation may be attributed to the influx of inflammatory cells into the arthritic joint, thereby changing the cell population to include cells with higher basal metabolic activity than the normal resident synoviocytes. This hypothesis is supported by the histological findings in our companion paper, where we found enhancements in cellular density close to the cartilage and, as stated above, around the suprapatellar bursa.

Methotrexate has, in the present dosage, antiinflammatory effects in the joint[18]. Our results with no marked effect on [^{3}H]-thymidine incorporation indicate that this dose of the drug does not markedly inhibit cell proliferation.

Blood flow and glucose metabolism in epiphyseal bone.

RBF in the epiphyseal bone was reduced in the arthritic knee on the first day of arthritis, then increased steadily, and was significantly elevated on Days 7 and 14, indicating that joint inflammation affects the bone. We have no obvious explanation for the initial decrease; it is possible that it represents a steal phenomenon. During the last 2 days of measurement the autoradiographic estimations of glucose uptake indicate that, in contrast to the findings in the soft tissue, the RBF is more elevated than the glucose uptake. This comparison thus supports the view that the blood flow in the soft tissue has a tendency to be insufficient.

In summary, our results indicate there is no increase in RBF during the first phase of arthritis despite a marked elevation in glucose metabolism. It remains possible that increased plasma extravasation has an attenuating effect on RBF. There is marked synovial hyperplasia characterized by an enhanced proliferation during the first days of arthritis. Most of the extra glucose metabolism is required for anaerobic glycolysis or processes other than cell proliferation, possibly related to the increased number of inflammatory cells. The main foci of the inflammation seem to be in the areas adjacent to cartilage.

REFERENCES

1. Falchuck KH, Goetzl EJ, Kulka JP. Respiratory gases of synovial fluid. Am J Med 1970;49:223-31.
2. Geborek P, Saxne T, Pettersson H, Wollheim FA. Synovial fluid acidosis correlates with radiological destruction in rheumatoid arthritis knee joints. J Rheumatol 1989;16:468-72.
3. Levick JR. Hypoxia and acidosis in chronic inflammatory arthritis; relation to vascular supply and dynamic effusion pressure [editorial]. J Rheumatol 1990;17:579-82.
4. Schumacher HR, Kitridou RC. Synovitis of recent onset. Arthritis Rheum 1972;15:465-85.
5. James MJ, Cleland LG, Rofe AM, Leslie AL. Intraarticular pressure and the relationship between synovial perfusion and metabolic demand. J Rheumatol 1989;17:521-7.
6. Blake DR, Unsworth J, Outhwaite JM, et al. Hypoxic-reperfusion injury in the inflamed joint. The Lancet 1989;8633:289-92.
7. Goetzl EJ, Falchuck KH, Zeiger LS, et al. A physiological approach to the assessment of disease activity in rheumatoid arthritis. J Clin Invest 1971;50:1167-80.
8. Palmer WE, Rosenthal DI, Schoenberg OI, et al. Quantification of inflammation in the wrist with gadolinium-enhanced MR imaging and PET with 2-(F-18)-fluoro-2-deoxy-D-glucose. Radiology 1995;196:647-55.
9. Winrow VR, Winyard PG, Morris CJ, Blake DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. Br Med Bull 1993;49:506-22.
10. Krenn V, Vollmers HP, v Ladenberg P, et al. Immortalized B-lymphocytes from rheumatoid synovial tissue show specificity for bacterial HSP 60. Virchows Arch 1996;427:511-8.
11. Balin AK, Fisher AJ, Carter DM. Oxygen modulates growth of human cells at physiologic partial pressures. J Exp Med 1984;160:152-66.
12. Graeber TG, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature 1996;379:88-91.
13. Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR. Somatic mutations in the p53 tumor supressor gene in rheumatoid arthritis synovium. PNAS 1997;94:10895-900.
14. Andersson SE, Ekström GM. Changes in vascular porosity and joint blood flow during the development of collagen-induced arthritis in the rat. Modulation by indomethacin and L-NAME. J Rheumatol 1997;24:2188-95.
15. Verschure PJ, Van Noorden CJF, Dijkstra CD. Macrophages and dendritic cells during the early stages of antigen-induced arthritis in rats: Immunohistochemical analysis of cryostat sections of whole knee joint. Scand J Immunol 1989;29:371-81.
16. Griffiths RJ. Characterisation and pharmacological sensitivity of antigen arthritis induced by methylated bovine serum albumin in the rat. Agents Actions 1992;35:88-95.
17. Carpenter MA, Everett LD, Hall MA, et al. High-resolution magnetic resonance imaging of arthritic pathology in the rat knee. Skeletal Radiol 1994;23:429-37.
18. Andersson SE, Lexmüller K, Ekström GM. Physiological characterization of mBSA antigen induced arthritis in the rat. I. Vascular leakiness and pannus growth. J Rheumatol 1998; 25;1772–7.
19. Wagner Jr HN, Rhodes BA, Sasaki Y, Ryan JP. Studies of the circulation with labelled microspheres. Invest Radiol 1969; 4:374-86.
20. Hillerdal M. Cochlear blood flow in the rat. A methodological evaluation of the microsphere method. Hearing Res 1984;27:27-35.
21. Hillerdal M, Sperber GO, Bill A. The microsphere method for measuring low blood flows: theory and computer simulations applied to findings in the rat cochlea. Acta Physiol Scand 1987;130:229-35.
22. Sokoloff L, Reivich M, Kennedy C, et al. The [C14] deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anaesthetized rat. J Neurochem 1977;28:897-916.
23. Ullberg S. The technique of whole body autoradiography. Cryosectioning of large specimens. In: Science Tools [special issue]. Bromma, Sweden: The LKB Instrument Journal; 1977:1-29.
24. Gondolf KB, Batsford S, Lässle G, Curschellas E, Mertz A. Handling of cationic antigens in the joint and induction of chronic allergic arthritis. Virchows Archiv B Cell Pathol 1991;60:353-63.
25. Andersson SE, Johansson A, Bjelke B, Ekström G. Characterisation of inflammatory activity in antigen-induced arthritis in the rat [abstract]. Ann Med Interne 1997;148:88.
26. Qu Z, Hernandez Garcia C, O'Rourke LM, et al. Local proliferation of fibroblast-like synoviocytes contributes to synovial hyperplasia. Arthritis Rheum 1994;37:212-20.
27. Jackson JR, Minton JAL, Ho ML, Wei N, Winkler JD. Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1ß. J Rheumatol 1997; 24:1253-9.
28. Hernvann A, Aussel C, Cynober L, Ekindijan OG. IL-1ß, a strong mediator for glucose uptake by rheumatoid and non-rheumatoid cultured human synoviocytes. FEBS 1992;303:77-80.
29. van de Loo AAJ, Arntz OJ, Bakker AC, van Lent PLEM, Jacobs MJM, van den Berg WB. Role of interleukin 1 in antigen-induced exacerbations of murine arthritis. Am J Pathol 1995;146:239-49.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-4

This is Exhibit SMT-4 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Toni Lee

JUSTICE OF THE PEACE (QUALIFIED)
DEPT. OF JUSTICE & ATTORNEY-GENERAL
No. 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

doi:10.1006/jaut.2001.0534, available online at http://www.idealibrary.com on IDEAL® Journal of Autoimmunity (2001) 17, 127–136

JAI

Systemic Macrophage Activation in Locally-induced Experimental Arthritis

Jutta Simon[1], Ralf Surber[2], Gesa Kleinstäuber[1], Peter K. Petrow[1], Steffen Henzgen[3], Raimund W. Kinne[4] and Rolf Bräuer[1]

[1]*Institute of Pathology, Friedrich Schiller University, Jena, Germany*
[2]*Department of Internal Medicine III, Friedrich Schiller University, Jena, Germany*
[3]*Institute of Pathology, Clinical Centre, Neubrandenburg, Germany*
[4]*Experimental Rheumatology Unit, Interdisciplinary Centre of Clinical Research, Friedrich Schiller University, Jena, Germany*

Received 27 March 2001
Accepted 7 June 2001

Key words: antigen-induced arthritis, macrophages, tumour necrosis factor-α, interleukin-1β, interleukin-6, interleukin-12, nitric oxide

Local and systemic macrophage activation was examined during the course of monoarticular murine antigen-induced arthritis (AIA), induced by systemic immunization and subsequent local induction. The levels of tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12p70, and nitric oxide (NO) were determined in joints, sera, and supernatants of peritoneal macrophages (the latter unstimulated or stimulated *ex vivo* with LPS/IFN-γ). In comparison with normal mice, systemic immunization (day 0) was associated to significant rise of TNF-α in serum, IL-1β in the joints, IL-6 in unstimulated macrophages and IL-12p70 in stimulated macrophages. Local induction led to a further significant increase of: (i) TNF-α, IL-1β, and IL-6 in the joints; and (ii) IL-1β, and IL-6 in sera and stimulated macrophages during acute and/or early chronic AIA (days 1 to 7). Unstimulated macrophages showed increased NO release (day 3), while stimulated macrophages significantly increased secretion of IL-12p70 (day 1). In late chronic AIA (day 21), cytokine/NO expression returned to immunization levels or below at all sites; solely IL-1β in the joints remained significantly above normal levels. Therefore, the prevalently local AIA model is characterized by a mixture of local and systemic activation of the mononuclear phagocyte system (MPS). While systemic MPS activation preceding arthritis induction can be attributed to systemic immunization, further systemic activation during arthritis appears an integral pathogenetic component of AIA.

Introduction

Human rheumatoid arthritis (RA) is a chronic systemic disorder of unknown aetiology, characterized by progressive joint destruction. Crucial pathogenic phenomena are joint inflammation, synovial hyperplasia, and abnormal immune responses [1], with infiltration of T-helper (Th) cells and macrophages into synovial tissue (reviewed in [2–4]). Antigen-induced arthritis (AIA), an intensively-studied experimental arthritis model, shows homologies to human RA in terms of histopathology and responses to several immunomodulatory drugs [5]. AIA is induced by systemic immunization with antigen (methylated bovine serum albumin; mBSA) in complete Freund's adjuvant (CFA), followed by single intra-articular injection of the antigen into the knee joint cavity [6].

Several lines of evidence suggest that activated macrophages play a relevant role in arthritis through: (i) processing and presentation of (auto)antigens to T cells; (ii) production of the pro-inflammatory mediators; tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, nitric oxide (NO) and other radicals; and (iii) production of tissue-degrading enzymes (reviewed in [3, 4]). TNF-α and IL-1, for example, may crucially affect mechanisms of joint destruction [7] or reactivate AIA [8]. IL-6 can act synergistically with IL-1 in the acute-phase reaction during inflammation, activating T cells and inducing terminal differentiation of B cells into plasma cells [9]. Activated macrophages also produce IL-12, a cytokine that critically influences the Th1/Th2 balance in favour of a Th1 pro-inflammatory activity (reviewed in [10]) and has a pro-arthritogenic role [11], at least partially in an autocrine fashion.

Finally, upregulation of the inducible isoform of nitric oxide synthase (iNOS), induced in macrophages and synoviocytes by various stimuli [12, 13], results in increased formation of NO; this mediator inhibits the synthesis of proteoglycans and is elevated in the synovial fluid of RA patients [14, 15].

However, the occurrence of systemically-secreted cytokines and their influence on locally-induced arthritis remains to be elucidated. This study focussed on the longitudinal course of systemic and local

Correspondence to: Prof. Dr Rolf Bräuer, Institut für Pathologie, Klinikum der Friedrich-Schiller-Universität, Ziegelmühlenweg 1, D-07740 Jena, Germany. Fax: 0049-3641-933111. E-mail: Braeuer@bach.med.uni-jena.de

0896–8411/01/060127+10 $35.00/0

cytokines prevalently produced by macrophages in murine AIA. Although a contribution of other cell types (in particular synovial fibroblasts and chondrocytes) to the cytokine levels cannot be excluded, the expression of these mediators was interpreted as a sign of macrophage activation. The advantage of AIA over other animal models lies in the exactly-defined initiation of arthritis, elicited by antigen injection into the knee joint cavity. The analyses included the acute and chronic stages of disease, as well as the preceding immunization status. Normal mice served as controls. Local macrophage activation was assessed by analysis of the pro-inflammatory mediators TNF-α, IL-1, IL-6, IL-12p70 (the active form of IL-12, a candidate stimulus of macrophage activation), and NO in the inflamed joints, while the serum and peritoneal macrophages harvested from the same animals (both unstimulated and after *ex vivo* stimulation) were analysed for signs of systemic activation.

Materials and Methods

Animals

Female C57BL/6 mice, 7–9 weeks of age, and male/female C3H/J mice, 4–8 weeks of age, were obtained from the Animal Research Facility, Beutenberg Campus, Friedrich Schiller University, Jena, Germany. They were housed under standard conditions, in a 12-h light/dark cycle, and fed with standard pellets (Altromin #1326, Lage, Germany) and water *ad libitum*. All animal studies were approved by the governmental commission for animal protection.

Induction of arthritis

On days −21 and −14, C57BL/6 mice were immunized by subcutaneous injection of 100 µg methylated bovine serum albumin (mBSA) in 50 µl saline, emulsified in 50 µl complete Freund's adjuvant (Sigma, Deisenhofen, Germany; adjusted to a concentration of 2 mg/ml of heat-killed *Mycobacterium tuberculosis*, strain H37RA/ Difco, Detroit, MI, USA). In addition, intraperitoneal injection of 2×10^9 heat-killed *Bordetella pertussis* (Pertussis Reference Center, Krankenhaus Berlin-Friedrichshain, Germany) was performed. Arthritis was elicited on day 0 by sterile injection of 100 µg mBSA in 25 µl saline into the right knee joint cavity, while the left knee joint remained untreated. Animals were analyzed before (non-arthritic mice systemically immunized with mBSA; day 0) and at various time-points after AIA induction (days 1, 3, 7, and 21). Normal mice served as controls. All measurements were performed in at least three independent experimental series, yielding comparable results.

Joint swelling

Mice were anaesthetized before immunization (normal), before arthritis induction (day 0), and on days 1, 3, 7, and 21 of AIA. The knee joint diameter was measured using an Oditest vernier calibre (Kroeplin Längenmesstechnik, Schlüchtern, Germany). Joint swelling was expressed as the difference in diameter (mm) between the right (arthritic) and left (control) knee joint.

Histology and grading of arthritis

After sacrificing the mice, both knee joints were removed *in toto*, skinned, and fixed in 4.5% phosphate-buffered formalin. After decalcification in EDTA, the joints were embedded in paraffin, cut into 5-µm-thick frontal sections, and stained with haematoxylin–eosin or safranin O for microscopic examination. The extent of joint inflammation (as defined by density of leukocytes infiltrated in the synovial membrane/joint space and degree of synovial lining layer hyperplasia) and the degree of joint destruction (cartilage necrosis, bone erosion, and pannus formation) were examined in four sections per knee joint by two independent observers (P.K.P., S.H.) and graded blindly using a semiquantitative score with 0=no, 1=mild, 2=moderate, and 3=severe alterations.

Analysis of serum cytokines

Mice were anaesthetized and killed by cervical dislocation. Sera were collected by total bleeding from the carotid artery and stored at −70°C. Analysis of murine TNF-α, IL-1β, IL-6, and IL-12p70 was performed with commercially available ELISA kits according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). The sensitivity of the assays was 5 pg/ml for murine TNF-α, 3 pg/ml for IL-1β or IL-6, and 2.5 pg/ml for IL-12p70.

Cytokine analysis in joint extracts

Whole joints were taken at various time points of AIA development, snap-frozen in isopropane/liquid nitrogen, and stored at −70°C until further analysis. Joint extracts were obtained according to Smith-Oliver *et al.* [16], i.e., the frozen joints were ground under liquid nitrogen with a mortar and pestle. The powdered tissue was transferred to a glass homogenizer, and exactly 2 ml of sterile saline were added. The powder suspension was homogenized by hand for 2 min and centrifuged for 20 min at $1500\times g$ and 4°C. The supernatant was spun again at $300\times g$ for 10 min, and the resulting supernatant aliquoted and frozen at −70°C. The assays proceeded using commercially available ELISA kits for murine TNF-α, IL-1β, IL-6, and IL-12p70 (R&D Systems; see above).

Isolation and stimulation of peritoneal macrophages

Peritoneal cells were harvested by peritoneal lavage of six to eight individual C57BL/6 mice with 7 ml cold phosphate-buffered saline (PBS) containing 5 IU/ml heparin (Liquemin N 2000, Hoffmann-La Roche, Grenzach-Whylen, Germany), before and at various times after arthritis induction. The washed cells were resuspended at a density of 1×10^6/ml in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), 100 U/ml penicillin (Jenapharm, Jena, Germany), 100 µg/ml streptomycin (Grünenthal, Stolberg, Germany), 0.5 µM 2-mercaptoethanol (Gibco), and 10 mM Hepes (Gibco; hereafter complete RPMI 1640 medium). A total of 1×10^6 cells/ml/well were seeded in 24-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany), and allowed to adhere for 2 h at 37°C and 5% CO_2. After attachment, non-adherent cells were removed by washing with warm medium and recounted to calculate the number of adherent cells in the well by subtraction. The remaining adherent cells consisted of >95% macrophages [17]. Following incubation for 24 h with or without the combination of 1 µg/ml LPS (*E. coli*, serotype O26:B6; Sigma) and 1 ng/ml recombinant murine IFN-γ (R&D Systems), the cell culture supernatants were removed, frozen in aliquots, and stored at −70°C until analysis of cytokines by bioassays (TNF-α, IL-1, and IL-6) or ELISA (IL-12p70), as well as NO analysis by the Griess reaction. For TNF-α determination, the cells were cultured in serum-free macrophage medium (Macrophage-SFM, Gibco). LPS-contamination of all media and solutions was <1 ng/ml (LAL-test; E-Toxate, Sigma).

TNF bioassay

TNF activity in culture supernatant of peritoneal macrophages was determined by a cytotoxicity bioassay, using the L929 fibroblast cell line [18]. Briefly, 5×10^4 L929 cells were plated in 96-well flat-bottom plates (Greiner Bio-One), duplicates incubated for 20 h with serial dilutions of test supernatant or recombinant murine (rm) TNF-α (R&D Systems), followed by viability assessment with crystal violet staining. The TNF-α concentration in the test samples was calculated by comparing the dilutions of rmTNF-α and the test samples inducing 50% cytolysis. Assay sensitivity was determined as 2 pg/ml murine TNF-α. Assay specificity was demonstrated by complete blocking of rmTNF-α activity (5 pg/ml) by addition of 100 pg/ml anti-TNF mAb (clone V1q).

IL-1 bioassay

IL-1 activity in culture supernatants was determined by a bioassay using co-stimulation of murine thymocytes [19]. Briefly, thymocytes from C3H/J mice were suspended at a density of 1×10^7/ml in complete RPMI 1640 medium. Duplicates (1×10^6 cells/well) were cultured in 96-well flat-bottom plates (Greiner Bio-One) for 70 h at 37°C and 5% CO_2, in the presence of 1 µg/ml concanavalin A (ConA; Pharmingen, Heidelberg, Germany) and either serially diluted (2-fold) macrophage supernatant or rmIL-1β (R&D Systems). ^{3}H-thymidine (0.5 µCi; specific activity 29 Ci/mmol; Amersham Buchler, Braunschweig, Germany) was added to each well for the last 22 h of incubation. The cells were harvested and the thymidine incorporation was measured. The IL-1 concentration in test samples was calculated by comparing the dilutions of rmIL-1β and the test samples inducing a 4-fold increase of thymidine incorporation. Assay sensitivity was 10 pg/ml IL-1β. In this bioassay, both IL-1α and IL-1β are biologically active, therefore the test sample concentrations were specified as IL-1.

IL-6 bioassay

IL-6 activity in culture supernatants was determined using the IL-6-dependent B9 cell line as described previously [20]. Briefly, 2.5×10^3 B9 cells (duplicates) were plated in 96-well plates (Greiner Bio-One), then serial dilutions of test supernatant or rmIL-6 standard (R&D Systems) were added and the cells incubated for 72 h at 37°C. For the last 4 h, 50 µg (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) were added to each well. Cells were then lysed with 10% SDS in 50% dimethylformamide and the absorbance read at 570 nm. The IL-6 concentration in test samples was calculated by comparing the dilutions of rmIL-6 and the test samples inducing half-maximal proliferation of B9 cells. Sensitivity of the assay was determined as 100 pg/ml rmIL-6. The specificity was validated by showing that neutralizing antibodies abrogated all effects of rmIL-6.

Measurement of NO production

The concentration of nitrite, which is proportional to NO production, NOS activity [21], and NOS-mRNA expression [22], was determined as described previously [20]. Briefly, test samples (100 µl) were mixed with an equal volume of Griess reagent in a 96-well microtitre plate (Greiner Bio-One). Absorbance was read at 570 nm and the test sample nitrite concentrations were calculated using known concentrations of sodium nitrite ($NaNO_2$) as a standard. Assay sensitivity was 1 µmol/l $NaNO_2$.

Statistical analysis

The multi-group Kruskall Wallis test was used for statistical evaluation of the data. Only those parameters that revealed significant differences ($P\leq0.05$) were further analysed with the non-parametric Mann–Whitney *U*-test. A *P*-value of ≤0.05 was considered to be statistically significant. Analyses

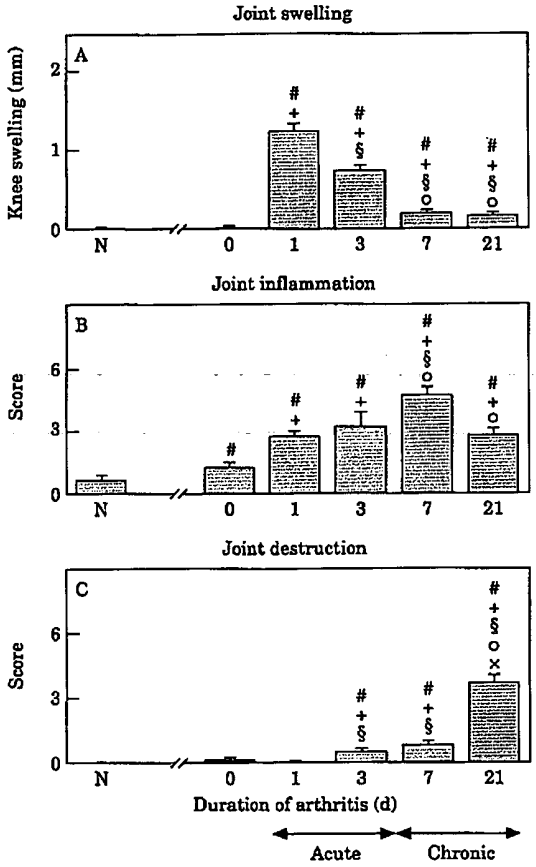


Figure 1. Time-course of AIA (days 0, 1, 3, 7, and 21) as measured by joint swelling (A) and histological scores of joint inflammation (B) and joint destruction (C) (see methods for details). Results are expressed as means±SEM of at least six individual animals per group. $^{\#}P \leq 0.05$, compared to normal control animals (N); $^{+}P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), $^{\S}P \leq 0.05$ compared to day 1, $^{\circ}P \leq 0.05$ compared to day 3, $^{\times}P \leq 0.05$ compared to day 7 of arthritis.

were performed using the SPSS 10.0® program (SPSS Inc., Chicago, IL, USA).

Results

Clinical disease activity

Joint swelling reached its maximum on day 1 of arthritis, and gradually declined until day 7 (Figure 1A). During the acute phase (days 1 and 3), joint swelling was significantly elevated compared to the chronic phase (days 7 and 21). In the chronic phase, the joint diameter remained significantly elevated in comparison with normal control mice and with mice which had only been systematically immunized (day 0; Figure 1A).

During arthritis, the joint inflammation score, as defined by hyperplasia of the synovial lining layer and cellular infiltration (Figure 1B), reached its maximum on day 7 and declined thereafter. In contrast, the joint destruction score, as defined by pannus formation, as well as cartilage and bone erosion, became significantly elevated on day 3 but reached its maximum only during late chronic arthritis (day 21; Figure 1C). Thus, the following phases could be identified: immunization (day 0), acute phase (days 1 to 3), early chronic (day 7) and late chronic phase (day 21).

Cytokine levels in joint extracts

In the immunization phase (day 0), therefore even before the injection of antigen into the joints, there was already a significant elevation of IL-1β compared to normal joints (Figure 2B). These, in turn, contained only trace amounts of IL-1β.

During the acute phase (days 1 and 3), the joint extracts displayed significant elevations of TNF-α, IL-1β, and IL-6 in comparison to the immunization phase (Figure 2A, B & C). On day 7, TNF-α and IL-6 returned to immunization levels (Figure 2A & C). IL-1β also significantly declined compared to day 3, however it remained significantly elevated compared with normal controls (Figure 2B). IL-1β remained significantly above normal controls also on day 21, i.e., well into the late chronic phase of AIA. In terms of degree of cytokine expression, the elevations of IL-1β and IL-6 were of the same order of magnitude (Figure 2B & C), reaching levels of approximately 1 ng/ml. In comparison, the elevation of TNF-α was moderate (Figure 2A), reaching only approximately 35 pg/ml.

Cytokine levels in the supernatants of AIA peritoneal macrophages

Unstimulated macrophages derived from the peritoneal cavity of AIA mice spontaneously released large quantities of IL-6 (approximately 20–30 ng/ml; Figure 2F), which significantly differed from those in normal mice throughout the course of AIA. The levels measured in samples obtained during AIA, however, were not significantly different from those observed upon systemic immunization (day 0; Figure 2F). Significant differences were instead observed between the acute (days 1 and 3) and late chronic phase of AIA (day 21; Figure 2F). There was no spontaneous release of TNF-α or IL-1 (Figure 2D & E).

Upon *ex vivo* stimulation with LPS *plus* IFN-γ, in contrast, AIA peritoneal macrophages produced significant levels of TNF-α and IL-1 (Figure 2G & H), i.e., significantly more than stimulated peritoneal macrophages derived from normal control mice (TNF-α, IL-1) or immunized mice (IL-1). Stimulated AIA peritoneal macrophages also significantly increased their production of IL-6 (Figure 2I). While in the case of TNF-α there was a single significant peak on day 1

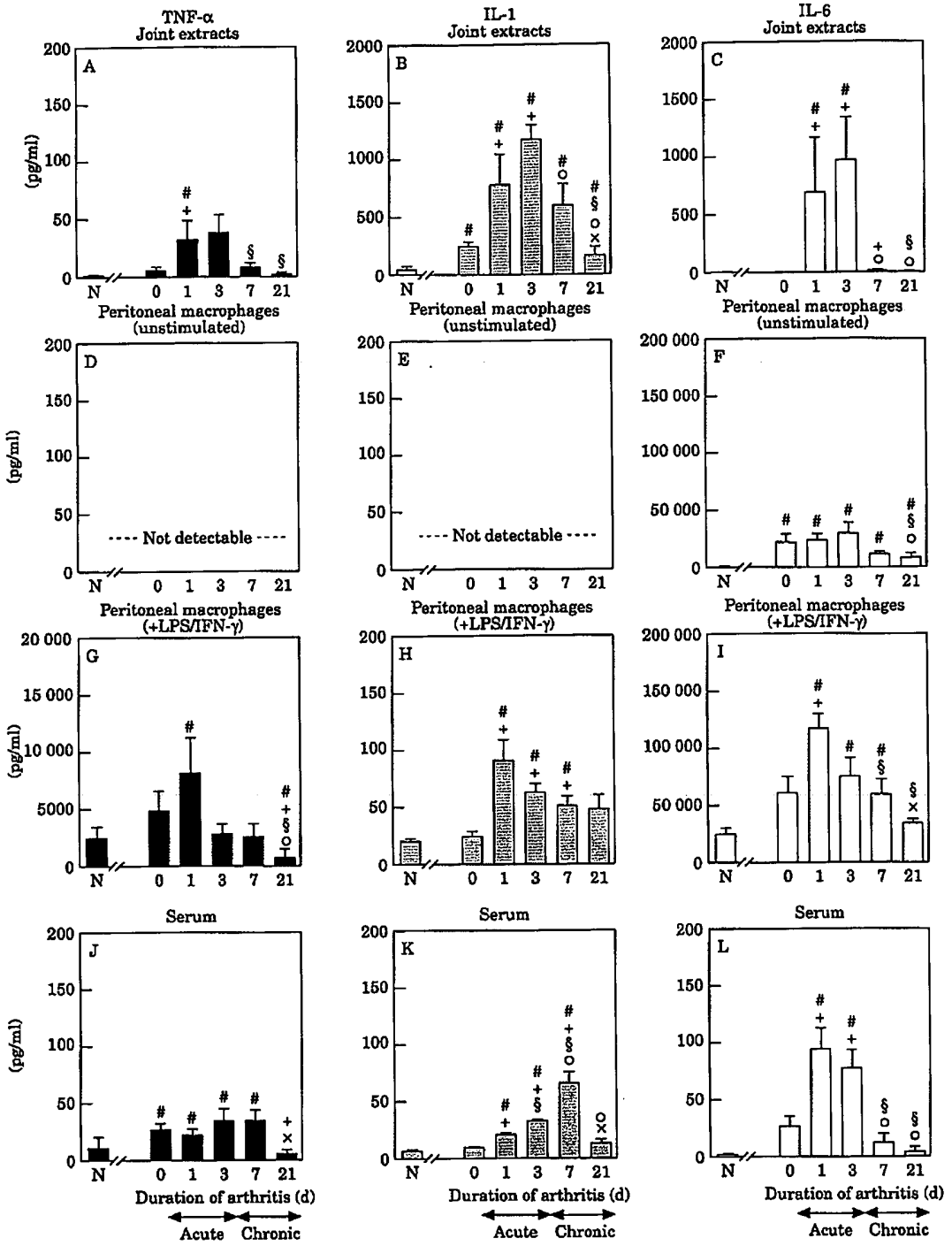


Figure 2. Cytokine concentrations (TNF-α, IL-1, and IL-6) in different compartments (joint extracts, A–C; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, D–I; and serum, J–L) during the course of AIA (days 0, 1, 3, 7, and 21) was measured by ELISA (joint extracts, sera) or bioassay (supernatants of macrophage cultures). Results are expressed as means±SEM of at least six individual animals per group. $^{\#}P \leq 0.05$, compared to normal control animals (N); $^{+}P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), $^{\S}P \leq 0.05$ compared to day 1, $^{\circ}P \leq 0.05$ compared to day 3, $^{\times}P \leq 0.05$ compared to day 7 of arthritis.

(Figure 2G), in the case of IL-1 and IL-6 the significant elevations extended to days 1, 3 and 7 (Figure 2H & I), matching the course of acute and early chronic disease (Figure 1A).

Cytokine levels in serum

The immunization status (day 0) was characterized by significant elevation of serum TNF-α (Figure 2J). A slight elevation of IL-6, in turn, was not significant compared to normal controls (Figure 2L). In the acute phase of arthritis, there was a significant elevation of TNF-α, IL-1β, and IL-6. For TNF-α, the elevations did not exceed those observed in immunized animals (day 0), maintaining a plateau between day 1 and day 7, and then dropping to normal control levels on day 21 (Figure 2J). Notably, the TNF-α elevation in the serum was of the same order of magnitude as that observed in joint extracts (Figure 2A), only more prolonged during the course of AIA (until day 7) and already visible upon immunization (day 0). In the case of IL-1β, the profile of the cytokine mount (significantly above normal and immunized mice) was opposite to that of the joint swelling (Figure 1A), as IL-1β slowly rose to reach a late peak only on day 7 (Figure 2K). On day 21, the IL-1β levels decreased significantly to normal control levels (Figure 2K). In the case of IL-6, significant peaks above normal and immunized mice were reached on day 1 and 3 of AIA. These declined significantly to normal levels on day 7 (Figure 2L).

IL-12p70

In the joints, minute amounts of IL-12p70 (<5 pg/ml) were detectable already in normal animals. These levels did not change upon immunization (day 0), but showed a minor, significant peak above normal levels during early acute AIA (day 1), with a significant decrease until day 7 (Figure 3A).

While resting peritoneal macrophages did not release any IL-12p70 (Figure 3B), *ex vivo* stimulated cells released considerable amounts of this cytokine (max. 250 pg/ml) (Figure 3C). The elevation was significant already upon immunization (day 0) in comparison with normal controls, but became more prominent on days 1 and 7 of AIA. Between these two time points, IL-12p70 dropped significantly on day 3 ($P<0.0001$; Figure 3C). This apparently biphasic course matched the peak of joint swelling on day 1 and appeared to accompany the transition into early chronicity (day 7; Figure 1A). This biphasic course was confirmed in three independent experimental series (data not shown).

Minute levels of IL-12p70 (max. 5 pg/ml) could also be detected in the serum of AIA mice, however they only minimally and non-significantly exceeded those of normal or immunized mice (Figure 3D).

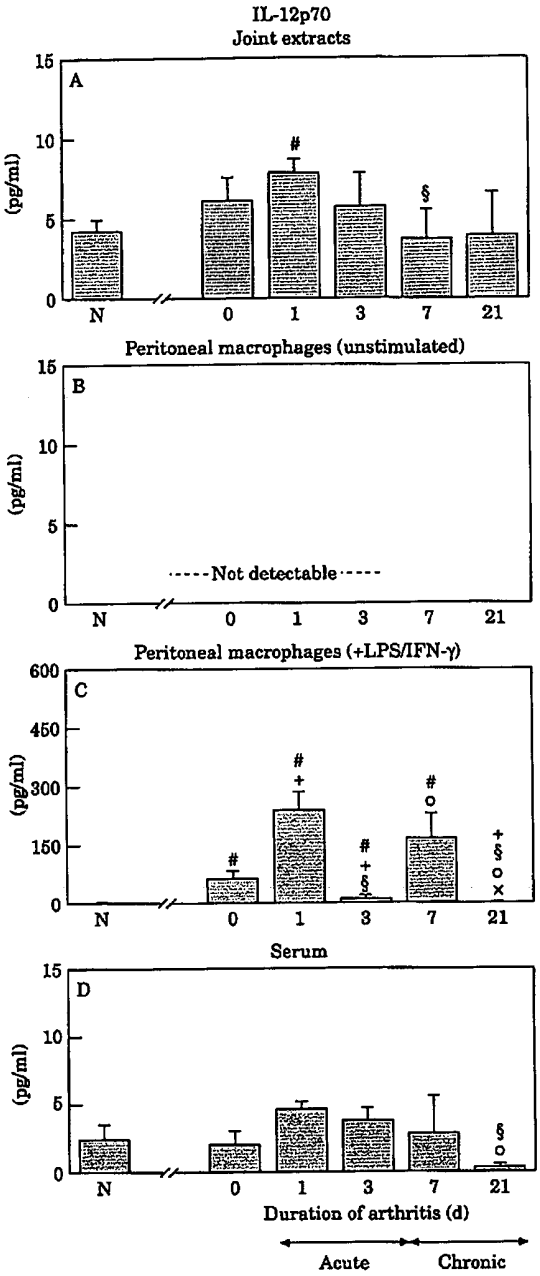


Figure 3. Concentration of IL-12p70 in different compartments (joint extracts, A; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, B–C; and serum, D) during the course of AIA (days 0, 1, 3, 7, and 21), as analysed by ELISA. Results are expressed as means±SEM of at least six individual animals per group. #$P\leq0.05$, compared to normal control animals (N); +$P\leq0.05$ compared to immunized, non-arthritic mice (day 0), §$P\leq0.05$ compared to day 1, °$P\leq0.05$ compared to day 3, ×$P\leq0.05$ compared to day 7 of arthritis.

Nitric oxide

NO was detectable only in the supernatants of AIA peritoneal macrophages. In unstimulated macrophages, the mean NO levels showed a significant peak above normal and immunized mice on day 3 (Figure 4B), while stimulated peritoneal macrophages only demonstrated a minor (1.5-fold), but significant peak above normal levels on day 1 of AIA, with a significant decrease thereafter (Figure 4C). The negative results in joint extracts (Figure 4A) and serum (Figure 4D) were not explainable by the presence of inhibitors of the Griess reaction in the respective compartments, since the addition of joint extracts or serum samples to known NO standards did not influence the expected results (data not shown).

Discussion

Cytokine profile during the immunization status

The immunization status immediately preceding the induction of local AIA (day 0) was characterized by signs of systemic macrophage activation, i.e., elevation of TNF-α in the serum and considerable release of IL-6 and IL-12p70 by peritoneal macrophages. The systemic involvement of macrophages is well-compatible with an MPS response to the spread of antigen and/or adjuvant from the injection site, in addition to a response to intraperitoneally-injected adjuvant (*Bordetella pertussis*).

Interestingly, at this preclinical stage cytokine production was also evident locally in the joints, as shown by significant elevations of IL-1β in comparison to normal. This partial macrophage activation at the local site is probably the result of repeated leakage of systemically-applied antigen into the joint [23], and is supported by local detection of IL-6 and IL-10 at this stage of murine AIA [24].

The source of serum TNF-α remains unclear. Because the serum levels at this stage clearly exceed the joint levels, a spill-over from the joints is highly unlikely. Previous studies in the prearthritis phase of other experimental arthritides [25, 26] map significant TNF-α and IL-1β mRNA production to the draining lymph nodes (but, for example, not the spleen), suggesting lymph nodes as a source of serum TNF-α. Whether elevated TNF-α production by activated circulating monocytes contributes to increased TNF-α levels in serum, in analogy to human rheumatoid arthritis [27], remains a matter of further investigation.

Cytokine profile during acute AIA

Acute AIA (days 1 and 3) was characterized by a significant rise of TNF-α, IL-1β, and IL-6 in the joints, as well as IL-1β and IL-6 in the serum, thus with a more complex cytokine pattern compared to the immunization phase.

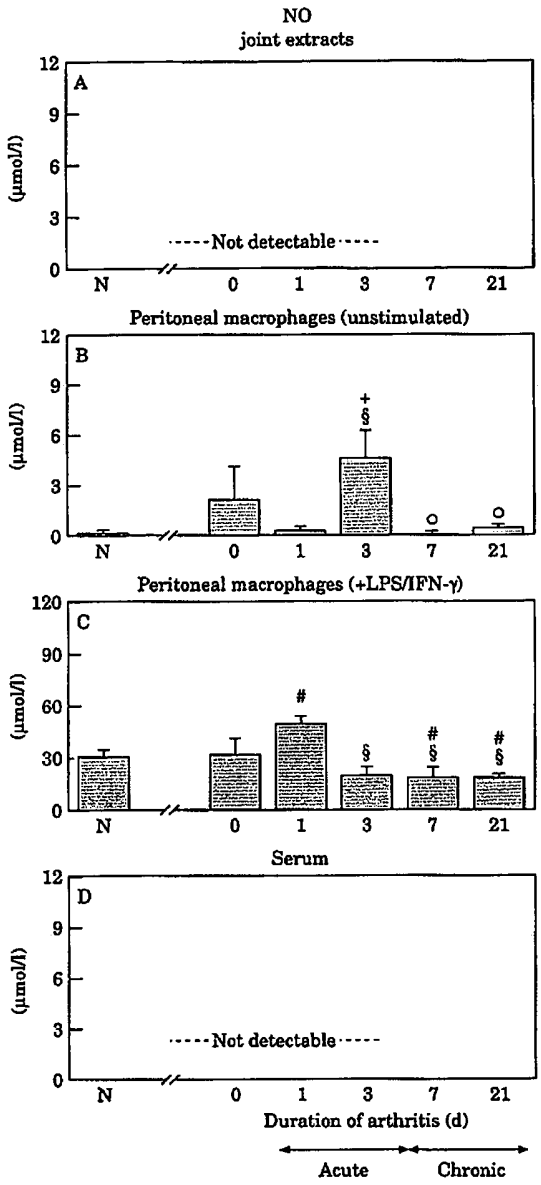


Figure 4. Nitrite concentration in different compartments (joint extracts, A; supernatants of unstimulated and *ex vivo* stimulated peritoneal macrophages, B–C; and serum, D) during the course of AIA (days 0, 1, 3, 7, and 21) was measured using the Griess reaction. Results are expressed as means±SEM of at least six individual animals per group. #$P \leq 0.05$, compared to normal control animals (N); *$P \leq 0.05$ compared to immunized, non-arthritic mice (day 0), §$P \leq 0.05$ compared to day 1, °$P \leq 0.05$ compared to day 3 of arthritis.

In the joint, the cytokine elevation matched the course of acute disease. However, the cytokine peak showed a 2-day delay in comparison with the peak of the joint swelling. These findings are well-consistent with the dynamics of the cellular infiltration, especially of macrophages [28], and stress the

importance of macrophage-derived cytokines in acute arthritis [24]. Quantitatively, TNF-α levels in the joints were approximately 25–40 times lower than those of IL-1β and IL-6. These findings stress the critical role of IL-1β and IL-6 in acute arthritis [3], and agree with the knowledge that in this animal model counteraction of IL-1β is more effective than counteraction of TNF-α [29]. On the other hand, the present data do not exclude that low TNF-α levels may be sufficient to influence the acute phase of experimental and human arthritis [25, 26, 30].

Presence and magnitude of IL-12p70 in joint extracts of AIA show good correspondence with data in experimental collagen-induced arthritis [31] and human RA [32, 33], and support a pathogenetic role for even small amounts of this cytokine also in AIA. Notably, the significant peak of IL-12p70 was reached on day 1, i.e. 2 days before the peak of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. This time course is compatible with a primary macrophage-activating role of IL-12 in AIA [11].

The lack of NO in the AIA joints, on the other hand, represents a clear difference in comparison with human RA [4] and experimental arthritides, such as AIA in rabbits [34] and rats [20]. Since the presence of inhibitors of the NO detection assay was excluded, the present findings probably reflect species differences in the AIA model.

In the serum, TNF-α elevation lasted until day 7, however at a constant level from the immunization phase. This stable course does not reflect the minor increase of this cytokine in the joints, and therefore suggests alternative production sites. At the equivalent stage of rat adjuvant arthritis, for example, TNF-α mRNA is expressed almost exclusively in the spleen [26]. IL-1β and IL-6, in contrast, showed serum peaks that generally matched the course of joint swelling, suggesting a possible spill-over from the joints. However, in the case of IL-1β, the peak levels were reached on day 7, i.e., 6 days after the joint swelling peak.

Whilst clear elevations of serum IL-6 have been previously observed in human RA [35, 36] and experimental arthritides including AIA [20, 37–39], elevated levels of IL-1 protein have been described in adjuvant arthritis [40], and, at least in some studies, in human RA [41, 42], but not in AIA [39]. Although the role of systemic elevation of IL-1β and IL-6 in AIA is presently unclear, evidence for a systemic pro-inflammatory influence of IL-6 on AIA has recently been reported in IL-6 knock-out mice [43].

Unstimulated peritoneal macrophages from AIA mice produced large amounts of IL-6 and low, but detectable amounts of NO, probably as a reaction to bacterial adjuvant and/or systemic spreading of antigen. In addition, peak production of IL-6 in unstimulated peritoneal macrophages, as well as TNF-α, IL-1, IL-6, and NO in LPS/IFN-γ-stimulated peritoneal macrophages, temporally coincided with or even preceded the time-course of swelling and cytokine production in AIA joints. This suggests parallel activation of macrophages in the joint and the peritoneal cavity during AIA, as previously observed in other experimental arthritis models [4]. Interestingly, IL-12p70 elevation in stimulated peritoneal macrophages was biphasic (days 1 and 7, with a significant drop on day 3), stressing that peritoneal macrophages may participate in the dual role of IL-12 during arthritis, i.e., disease promotion in early disease, but disease suppression in late phases [44].

Cytokine profile during chronic AIA

IL-1β was the only cytokine to remain significantly elevated in the joints during chronic AIA, similarly to findings in other arthritis models [40, 45]. The temporal coincidence with the progression of joint destruction, starting in the early chronic phase (see Figure 1C), is compatible with a central importance of this cytokine for the destruction of cartilage and bone in experimental arthritis [46, 47]. Parallel, selective elevation of IL-1β in joints, macrophage supernatants, and serum (although at clearly reduced levels compared to the acute phase), suggests that macrophage may primarily use IL-1β as a mediator for their functions in chronic disease [26]. This is in clear contrast to the simultaneous elevation of all investigated cytokines in the acute phase of AIA.

General considerations

In spite of its local character, AIA exhibits clear systemic signs of macrophage activation, i.e., the elevation in the serum of the pro-arthritogenic cytokines TNF-α and IL-1β, as well as the increased levels of IL-6, a cytokine involved in the acute phase response circuit. Also, peritoneal macrophages show signs of activation in AIA, both constitutively and upon additional *ex vivo* stimulation. Since the systemic signs appearing in acute AIA mostly exceed those observed upon systemic immunization, systemic MPS activation appears to be an integral pathogenetic feature of this local arthritis model, presumably driven by leakage of antigen and pro-inflammatory stimuli from the inflamed joints. This systemic feature of arthritis can clearly be observed also in human RA [27, 48, 49], and makes systemic counteraction of MPS activation an attractive target for therapeutic intervention [50, 51].

Acknowledgements

Dr Ernesta Palombo-Kinne is gratefully acknowledged for critical revision of the manuscript, and Waltraud Kröber and Uta Griechen for excellent technical assistance. This work was supported by the German Federal Ministry of Education and Research (Grant FKZ 01ZZ9602 to R.B. and R.W.K.) and the Deutsche Forschungsgemeinschaft (Grant Br 1372/2 to R.B.).

References

1. Gay S., Gay R.E., Koopman W.J. 1993. Molecular and cellular mechanisms of joint destruction in rheumatoid

arthritis: Two cellular mechanisms explain joint destruction? *Ann. Rheum. Dis.* (suppl.) 52: 39–47

2. Fox D.A. 1997. The role of T cells in the immunopathogenesis of rheumatoid arthritis: New perspectives. *Arthritis Rheum.* 40: 598–609
3. van den Berg W.B., van Lent P.L. 1996. The role of macrophages in chronic arthritis. *Immunobiology* 195: 614–623
4. Kinne R.W., Bräuer R., Stuhlmüller B., Palombo-Kinne E., Burmester G.R. 2000. Macrophages in rheumatoid arthritis. *Arthritis Res.* 2: 189–202
5. Crossley M.J., Holland T., Spowage M., Hunneyball I.M. 1989. Monoarticular antigen-induced arthritis in rabbits and mice. *Modern Methods Pharmacol.* 5: 415–439
6. Brackertz D., Mitchell G.F., Mackay I.R. 1977. Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice. *Arthritis Rheum.* 20: 841–850
7. Bondeson J. 1997. The mechanisms of action of disease-modifying antirheumatic drugs: A review with emphasis on macrophage signal transduction and the induction of proinflammatory cytokines. *Gen. Pharmacol.* 29: 127–150
8. van de Loo A.A., Arntz O.J., van den Berg W.B. 1992. Flare-up of experimental arthritis in mice with murine recombinant IL-1. *Clin. Exp. Immunol.* 87: 196–202
9. Graeve L., Baumann M., Heinrich P.C. 1993. Interleukin-6 in autoimmune disease. Role of IL-6 in physiology and pathology of the immune defense. *Clin. Invest.* 71: 664–671
10. Germann T., Rüde E. 1995. Interleukin-12. *Int. Arch. Allergy Immunol.* 108: 103–112
11. Germann T., Szeliga J., Hess H., Storkel S., Podlaski F.J., Gately M.K., Schmitt E., Rüde E. 1995. Administration of interleukin 12 in combination with type II collagen induces severe arthritis in DBA/1 mice. *Proc. Natl. Acad. Sci. USA* 92: 4823–4827
12. Marletta M.A. 1993. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* 268: 12231–12234
13. Stefanovic-Racic M., Stadler J., Evans C.H. 1993. Nitric oxide and arthritis. *Arthritis Rheum.* 36: 1036–1044
14. Moilanen E., Vapaatalo H. 1995. Nitric oxide in inflammation and immune response. *Ann. Med.* 27: 359–367
15. Gibaldi M. 1993. What is nitric oxide and why are so many people studying it? *J. Clin. Pharmacol.* 33: 488–496
16. Smith-Oliver T., Noel L.S., Stimpson S.S., Yarnall D.P., Connolly K.M. 1993. Elevated levels of TNF in the joints of adjuvant arthritic rats. *Cytokine* 5: 298–304
17. Yui S., Sasaki T., Yamazaki M. 1993. Augmentation and suppression of TNF release from macrophages by inflammatory polymorphonuclear leukocytes. *Microbiol. Immunol.* 37: 801–808
18. Feist W., Ulmer A.J., Wang M.H., Musehold J., Schlüter C., Gerdes J., Herzbeck H., Brade H., Kusumoto S., Diamantstein T., Rietschel E.T., Flad H.D. 1992. Modulation of lipopolysaccharide-induced production of tumor necrosis factor, interleukin 1, and interleukin 6 by synthetic precursor Ia of lipid. *FEMS Microbiol. Immunol.* 4: 73–89
19. Foresta P., Ruggiero V., Albertoni C., Pacello L., Leoni B., Arrigoni Martelli E. 1992. In vitro activation of murine peritoneal exudate cells (PEC) and peritoneal macrophages by ST 789. *Int. J. Immunopharmacol.* 14: 1061–1068
20. Mentzel K., Bräuer R. 1998. Matrix metalloproteinases, IL-6, and nitric oxide in rat antigen-induced arthritis. *Clin. Exp. Rheumatol.* 16: 269–276
21. Severn A., Xu D., Doyle J., Leal L.M., O'Donnell C.A., Brett S.J., Moss D.W., Liew F.Y. 1993. Pre-exposure of murine macrophages to lipopolysaccharide inhibits the induction of nitric oxide synthase and reduces leishmanicidal activity. *Eur. J. Immunol.* 23: 1711–1714
22. Lorsbach R.B., Murphy W.J., Lowenstein C.J., Snyder S.H., Russell S.W. 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-γ and lipopolysaccharide. *J. Biol. Chem.* 268: 1908–1913
23. van Lent P.L., van den Berg W.B., Schalkwijk J., van de Putte L.B., van den Bersselaar L. 1987. Allergic arthritis induced by cationic antigens: Relationship of chronicity with antigen retention and T-cell reactivity. *Immunology* 62: 265–272
24. Hersmann G.H.W., Kriegsmann J., Simon J., Hüttich C., Bräuer R. 1998. Expression of cell adhesion molecules and cytokines in murine antigen-induced arthritis. *Cell Adhes. Commun.* 6: 69–82
25. Müssener A., Klareskog L., Lorentzen J.C., Kleinau S. 1995. TNF-α dominates cytokine mRNA expression in lymphoid tissues of rats developing collagen- and oil-induced arthritis. *Scand. J. Immunol.* 42: 128–134
26. Schmidt-Weber C.B., Pohlers D., Siegling A., Schädlich H., Buchner E., Volk H.D., Palombo-Kinne E., Emmrich F., Kinne R.W. 1999. Cytokine gene activation in synovial membrane, regional lymph nodes, and spleen during the course of rat adjuvant arthritis. *Cell. Immunol.* 195: 53–65
27. Stuhlmüller B., Ungethüm U., Scholze S., Martinez L., Backhaus M., Kraetsch H.G., Kinne R.W., Burmester G.R. 2000. Identification of known and novel genes in activated monocytes from patients with rheumatoid arthritis. *Arthritis Rheum.* 43: 775–790
28. Dijkstra C.D., Dopp E.A., Vogels I.M., van Noorden C.J. 1987. Macrophages and dendritic cells in antigen-induced arthritis. An immunohistochemical study using cryostat sections of the whole knee joint of rat. *Scand. J. Immunol.* 26: 513–523
29. van de Loo F.A.J., Joosten L.A.B., van Lent P.L.E.M., Arntz O.J., van den Berg W.B. 1995. Role of interleukin-1, tumor necrosis factor-α, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of *in situ* blocking in murine antigen- and zymosan-induced arthritis. *Arthritis Rheum.* 38: 164–172
30. Alsalameh S., Winter K., Al-Ward R., Wendler J., Kalden J.R., Kinne R.W. 1999. Distribution of TNF-α, TNF-R55 and TNF-R75 in the rheumatoid synovial membrane: TNF receptors are localized preferentially in the lining layer; TNF-α is distributed mainly in the vicinity of TNF receptors in the deeper layers. *Scand. J. Immunol.* 49: 278–285
31. Yoshino S., Ohsawa M. 2000. The role of lipopolysaccharide injected systemically in the reactivation of collagen-induced arthritis in mice. *Br. J. Pharmacol.* 129: 1309–1314
32. Morita Y., Yamamura M., Nishida K., Harada S., Okamoto H., Inoue H., Ohmoto Y., Modlin R.L.,

Makino H. 1998. Expression of interleukin-12 in synovial tissue from patients with rheumatoid arthritis. *Arthritis Rheum.* **41**: 306–314

33. Kim W.U., Min S.Y., Cho M.L., Youn J., Min D.J., Lee S.H., Park S.H., Cho C.S., Kim H.Y. 2000. The role of IL-12 in inflammatory activity of patients with rheumatoid arthritis (RA). *Clin. Exp. Immunol.* **119**: 175–181
34. Palacios F.A., Novaes G.S., Guzzo M.L., Laurindo I.M., de Mello S.B. 1999. Interrelationship of the kinin system, nitric oxide and eicosanoids in the antigen-induced arthritis in rabbits. *Mediators Inflamm.* **8**: 245–251
35. Sack U., Kinne R.W., Marx T., Heppt P., Bender S., Emmrich F. 1993. Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis. *Rheumatol. Int.* **13**: 45–51
36. Ohshima S., Saeki Y., Mima T., Sasai M., Nishioka K., Ishida H., Shimizu M., Suemura M., McCloskey R., Kishimoto T. 1999. Long-term follow-up of the changes in circulating cytokines, soluble cytokine receptors, and white blood cell subset counts in patients with rheumatoid arthritis (RA) after monoclonal anti-TNF-α antibody therapy. *J. Clin. Immunol.* **19**: 305–313
37. Sasai M., Saeki Y., Ohshima S., Nishioka K., Mima T., Tanaka T., Katada Y., Yoshizaki K., Suemura M., Kishimoto T. 1999. Delayed onset and reduced severity of collagen-induced arthritis in interleukin-6-deficient mice. *Arthritis Rheum.* **42**: 1635–1643
38. Theisen-Popp P., Pape H., Müller-Peddinghaus R. 1992. Interleukin-6 (IL-6) in adjuvant arthritis of rats and its pharmacological modulation. *Int. J. Immunopharmacol.* **14**: 565–571
39. Bräuer R., Kette H., Henzgen S., Thoss K. 1994. Influence of cyclosporin A on cytokine levels in synovial fluid and serum of rats with antigen-induced arthritis. *Agents Actions* **41**: 96–98
40. Szekanecz Z., Halloran M.M., Volin M.V., Woods J.M., Strieter R.M., Haines G.K., Kunkel S.L., Burdick M.D., Koch A.E. 2000. Temporal expression of inflammatory cytokines and chemokines in rat adjuvant-induced arthritis. *Arthritis Rheum.* **43**: 1266–1277
41. Zoli A., Altomonte L., Caricchio R., Galossi A., Mirone L., Ruffini M.P., Magaro M. 1998. Serum zinc and copper in active rheumatoid arthritis: Correlations with interleukin 1β and tumor necrosis factor-α. *Clin. Rheumatol.* **17**: 378–382
42. Alstergren P., Ernberg M., Kopp S., Lundeberg T., Theodorsson E. 1999. TMJ pain in relation to circulating neuropeptide Y, serotonin, and interleukin-1β in rheumatoid arthritis. *J. Orofac. Pain* **13**: 49–55
43. Boe A., Baiocchi M., Carbonatto M., Papoian R., Serlupi-Crescenzi O. 1999. Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis. *Cytokine* **11**: 1057–1064
44. Joosten L.A., Lubberts E., Helsen M.M., van den Berg W.B. 1997. Dual role of IL-12 in early and late stages of murine collagen type II arthritis. *J. Immunol.* **159**: 4094–4102
45. Andersson S.E., Lexmüller K., Alving K., Ekström G.M. 1999. Periarticular tissue levels of cytokine- and endothelin-1-like immunoreactivity during the course of antigen-induced arthritis in the rat. *Inflamm. Res.* **48**: 491–496
46. Stasiuk L.M., Abehsira-Amar O., Fournier C. 1996. Collagen-induced arthritis in DBA/1 mice: Cytokine gene activation following immunization with type II collagen. *Cell. Immunol.* **173**: 269–275
47. Joosten L.A., Helsen M.M., Saxne T., van de Loo F.A., Heinegard D., van den Berg W.B. 1999. IL-1αβ blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-α blockade only ameliorates joint inflammation. *J. Immunol.* **163**: 5049–5055
48. Heller R.A., Schena M., Chai A., Shalon D., Bedilion T., Gilmore J., Woolley D.E., Davis R.W. 1997. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc. Natl. Acad. Sci. USA* **94**: 2150–2155
49. Schulze-Koops H., Davis L.S., Kavanaugh A.F., Lipsky P.E. 1997. Elevated cytokine messenger RNA levels in the peripheral blood of patients with rheumatoid arthritis suggest different degrees of myeloid cell activation. *Arthritis Rheum.* **40**: 639–647
50. Kinne R.W., Schmidt-Weber C.B., Hoppe R., Buchner E., Palombo-Kinne E., Nürnberg E., Emmrich F. 1995. Long-term amelioration of rat adjuvant arthritis following systemic elimination of macrophages by clodronate-containing liposomes. *Arthritis Rheum.* **38**: 1777–1790
51. Richards P.J., Williams A.S., Goodfellow R.M., Williams B.D. 1999. Liposomal clodronate eliminates synovial macrophages, reduces inflammation and ameliorates joint destruction in antigen-induced arthritis. *Rheumatology* **38**: 818–825

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-5

This is Exhibit SMT-5 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2009

Before me:

Doni Russo

JUSTICE OF THE PEACE (QUALIFIED) DEPT OF JUSTICE & ATTORNEY-GENERAL Reg. No. 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

Clin Exp Immunol 1997; 110:45–52

Local therapy with soluble complement receptor 1 (sCR1) suppresses inflammation in rat mono-articular arthritis

R. M. GOODFELLOW, A. S. WILLIAMS, J. L. LEVIN†, B. D. WILLIAMS & B. P. MORGAN* *Departments of Rheumatology and *Medical Biochemistry, University of Wales College of Medicine, Cardiff, UK, and †T Cell Sciences Inc., Needham, MA, USA*

(Accepted for publication 8 July 1997)

SUMMARY

Complement activation has been implicated in the pathogenesis of human rheumatoid arthritis. We sought to determine whether inhibition of complement (C) using sCR1 could influence the development and progression of antigen arthritis in the rat, a recognized model of human chronic synovitis. The effect of C inhibition, systemically and locally, on three different stages of disease was examined: (i) prophylaxis, (ii) treatment of established inflammation, and (iii) prevention of antigen-induced flares of disease. Arthritis was assessed by knee swelling and by histological examination. Our results show that intra-articular injection of sCR1 prior to disease onset reduced joint swelling and development of arthritis, whereas systemic administration was ineffective. Treatment of established arthritis with intra-articular sCR1 3 days after disease onset caused a transient reduction in swelling, but treatment 7 days after disease onset had no effect on disease. An intra-articular dose of sCR1 given at the time of disease flares had a small, yet significant effect on knee swelling. We conclude that complement activation is important in the initiation and maintenance of inflammation in antigen arthritis. The potent effect of local C inhibition suggests that C biosynthesis and activation within the joint contributes to inflammation in this model of arthritis.

Keywords antigen arthritis rat complement sCR1

INTRODUCTION

Activation of complement (C) has been shown to contribute to tissue damage in a variety of inflammatory and infectious diseases [1]. Evidence of an involvement of C in rheumatoid arthritis (RA) has come from several sources; C activation products are present in blood and synovial fluid of patients with active RA [2–7], and products of C activation are deposited in the rheumatoid synovium. Human rheumatoid synovial cells attacked *in vitro* with C are induced to release a spectrum of inflammatory products including prostaglandins and cytokines [8,9], providing a mechanism for the perpetuation of joint inflammation. The development of a recombinant soluble form of the human membrane complement regulatory molecule, complement receptor one (sCR1:TP10) has been described [10]. This 247-kD protein is a potent inhibitor of the classical and alternative complement pathways. It binds to C3b and C4b, resulting in inactivation of the C3 and C5 convertase. It has proved an excellent inhibitor of the *in vitro* activation of serum complement in humans and a variety of other species. Since its first description, sCR1 has been shown to suppress tissue injury in a variety of animal models of human disease, including myocardial [11] and gut [12] ischaemia/reperfusion injury, graft rejection [13], adult respiratory distress syndrome [14] and experimental allergic encephalomyelitis [15]. This study describes the effect of sCR1 on the clinical course and pathogenesis of rat antigen arthritis, an animal model of human chronic synovitis. We show that intra-articular injection of sCR1 markedly reduced joint swelling and development of destructive arthritis, the latter judged by assessing the neutrophil infiltrate into the synovium and erosion of articular cartilage. Systemic sCR1 caused little inhibition of this arthritis, and the combination of therapies was not significantly better than intra-articular therapy alone. These results suggest that C is involved in the generation of pathology in this model and suggest that intra-articular synthesis of C is important in the initiation and perpetuation of the inflammatory process.

Correspondence: Dr Rhian Goodfellow, Department of Rheumatology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK.

MATERIALS AND METHODS

Animals

Male Lewis rats (100–150 g) were obtained from Bantin and Kingman (The Field Station, Hull, UK) and housed in cages of

four at Biomedical Services U.W.C.M. They were allowed free access to food and water and kept in light/dark cycles of 12 h.

Arthritis induction

Arthritis was induced following an established method [16]. Briefly, on two occasions a week apart, an emulsion containing equal volumes of methylated bovine serum albumin (mBSA; 0·5 mg/ml; Sigma Chemical Co., St Louis, MO) and Freund's complete adjuvant (FCA; 0·25 mg *Mycobacterium tuberculosis*; Sigma) was injected subcutaneously into the backs of the animals. Fourteen days after this second subcutaneous injection (day 0), an intra-articular injection of mBSA (0·1 mg in 100 μl of saline) was given into the right knee of each animal. The left knee served as a control and received an equal volume of saline.

Treatment regimens

Cobra venom factor (CVF) was purified from the venom of the Naja Naja cobra (Sigma) by the method of Vogel & Muller-Eberhard [17] and was a gift from Dr J. P. Camilleri (U.W.C.M.).

Recombinant, endotoxin-free sCR1 (TP10) at a concentration of 4·95 mg/ml in sterile PBS pH 7·0 was provided by T Cell Sciences Inc. (Needham, MA).

The effect of C inhibition on three different stages of disease was examined: (i) prophylaxis, (ii) treatment of established inflammation, and (iii) prevention of antigen-induced flares of disease.

Prophylaxis. Animals were randomized into groups of five. Disease was induced in all animals. Each group of five received one of the following treatment regimens: (i) i.p. CVF 100 U 2 days before onset of disease; (ii) i.p. CVF 2 days before onset of disease and intra-articular sCR1 with the disease-initiating antigen; (iii) i.v. sCR1 20 mg/kg daily for 7 days starting 2 days before disease onset; (iv) i.v. and intra-articular sCR1; (v) intra-articular sCR1(200 μg) as a single dose with the disease-initiating antigen; (vi) control saline-treated group.

Treatment. Arthritis was induced as described above and animals were randomized into groups of five, each group of animals to receive one of the following regimens: (i) 200 μg of sCR1 given intra-articularly 3 days after disease onset; (ii) 200 μg sCR1 given intra-articularly 7 days after disease onset; (iii) untreated controls.

Flare reaction. Arthritis was induced as described previously. Fourteen days after disease onset a flare was induced in 10 animals by a second intra-articular injection of mBSA (0·1 mg) into the inflamed right knee. Animals were divided into two groups matched for disease severity, one group receiving an intra-articular dose of sCR1 ($n = 5$) with the boosting antigen, control rats ($n = 5$) receiving mBSA alone.

Haemolytic assays

The animals receiving systemic therapy were bled daily and the haemolytic activity of each plasma sample was measured by minor modifications of published methods to confirm complement inhibition [18]. Briefly, plasma samples were diluted in veronal buffer and incubated with a standard concentration of sheep erythrocytes sensitized with ambozeptor (rabbit anti-sheep globin; Behring Hounslow, UK). Results were expressed as a percentage of normal rat plasma haemolytic activity in the same system

Disease assessment

Clinical. Disease was assessed clinically by measuring the knee diameters of both inflamed (right) and non-inflamed (left) knees, with a Mitutoyo digital calliper. The measurements were done in triplicate by an independent observer blind to the treatment regimen. The swelling attributed to the antigenic challenge was expressed as the difference in millimetres between the mean readings of the right (inflamed) and left (normal) knee diameters.

Histological. Rats from different treatment groups were killed 14 days after arthritis induction. The knee joints were removed intact and fixed in formalin-buffered saline for 1 week before histological processing. Joints were decalcified and embedded in paraffin wax, then sectioned in the sagittal plane at 5 μm and stained with haematoxylin–eosin. All sections were coded before assessment to eliminate observer bias and subsequently scored by an independent observer. Sections were graded subjectively on a scale of 0–4: 0 = normal; 1 = minimal synovial infiltration; 2 = moderate synovial infiltrate with inflammatory exudate in joint space; 3 = severe synovial infiltration and inflammatory exudate; 4 = severe synovial infiltration, inflammatory exudate and full thickness cartilage destruction with bone erosion.

Statistical analysis

The one way analysis of variance (ANOVA) was used to determine the significance of differences between the knee diameters in different treatment groups. Student's *t*-test was used to determine the significance of differences between individual groups. The Wilcoxon sum of ranks test was used to determine the significance of differences in the histological scores of the knees.

RESULTS

Decomplementation with sCR1 and CVF

CVF given as a single i.p. dose reduced the haemolytic activity in serum to zero. The animals remained completely decomplemented for 5 days. In sCR1-treated animals haemolytic assays were performed 24 h after each dose. Haemolytic activity of serum was reduced to 60% of the haemolytic activity of serum at day 0. However, this is an underestimate of the level of decomplementation attained during the 24 h post-therapy. Figure 1 shows a clearance study in naive animals. The haemolytic activity of rat serum after a single i.v. dose of sCR1 (20 mg/kg per animal) fell rapidly to 20% of that of normal rat serum within the first 15 min post-i.v. injection, rose to 40% by 2 h, was maintained at this level

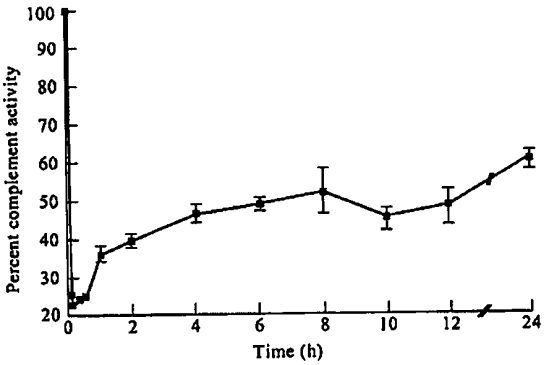


Fig. 1. Residual complement activity in rat serum after a single i.v. dose of sCR1 (20 mg/kg) over 24 h. Results are means of determinations in three naive rats, the vertical bar represents s.e.m. of the three measurements.

for a further 12 h and then slowly rose so that at 24 h the haemolytic activity was 60% that of normal rat serum. Animals receiving intra-articular sCR1 showed no evidence of systemic decomplementation (data not shown).

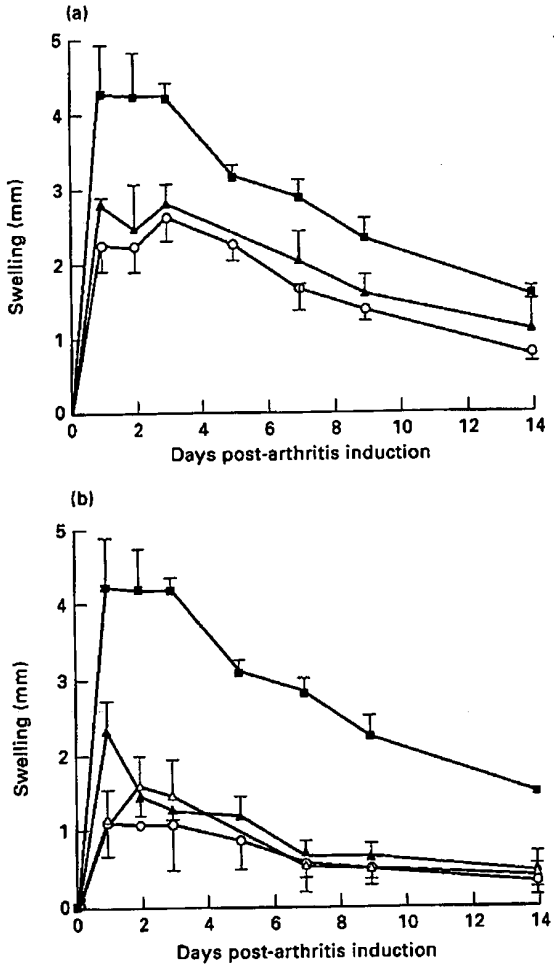


Fig. 2. (a) Effect of i.p. cobra venom factor (CVF) (▲), i.v. sCR1 (○), and saline (■) on the development of mono-articular arthritis in the Lewis rat. Significant difference ($P<0.05$) was seen in the knee swelling of the systemic CVF-treated group compared with controls on day 2; thereafter there was no significant difference. Similarly, significant difference was seen in the knee swelling in animals that received i.v. sCR1 compared with controls on day 1 ($P<0.05$), which became non-significant from day 7 until the end of the experiment. (b) Effect of intra-articular sCR1 (▲), i.v. and intra-articular sCR1 (○), i.p. CVF and intra-articular sCR1 (△) and saline (■) on the development of antigen arthritis in the Lewis rat. Results are means of five animals ± s.e.m. Significant differences were seen in the knee swelling of animals in all treated groups compared with controls throughout the experiment. There was no statistical difference between the knee swelling of animals in the groups that received systemic CVF or sCR1 in addition to intra-articular sCR1 and the group that received intra-articular therapy alone. Data are from a single study. The study was repeated three times with comparable results.

Effects of therapy on clinical disease

Prophylaxis. The effects on antigen arthritis of inhibition of C with either CVF or i.v. sCR1 are shown in Fig. 2a. CVF had a small but significant effect on the swelling in the first 2 days after antigenic challenge ($P<0.05$). Swelling was decreased compared with C-sufficient controls (2·75 mm compared with 4·2 mm). However, this effect was not sustained beyond 2 days, and the difference in swelling became non-significant for the remainder of the experiment. Intravenous sCR1 alone showed similar results, having a significant anti-inflammatory effect for 3 days.

The effects on antigen arthritis of intra-articular therapy with sCR1 are shown in Fig. 2b. A significant reduction in joint swelling in both groups that received intra-articular sCR1 was apparent 1 day after intra-articular challenge ($P<0.02$), which persisted for the duration of the experiment. The addition of systemic decomplementation using either CVF or sCR1 did not further enhance the anti-inflammatory effect of intra-articular therapy alone.

Treatment. Treatment of established arthritis with a single intra-articular dose of sCR1 3 days after disease onset caused a small, transient reduction in swelling which was significant at 24 h (Fig. 3). Treatment 7 days after disease onset, however, had no effect on the clinical disease (data not shown).

Treatment of a 'flare'. A repeat intra-articular challenge given 14 days after initiation of disease caused a flare in disease activity and increase in joint swelling (Fig. 4a). The flare was significantly dampened ($P<0.02$) in the group which received intra-articular sCR1 at the same time as the flare-inducing antigen. This difference persisted for 3 days after therapy. Identical results were seen when the flare was induced on established chronic disease, 40 days after disease initiation (Fig. 4b).

Histology

Table 1 summarizes the results of histological analyses of the fixed joint sections, and representative micrographs of the histological sections are shown in Fig. 5. The results mirror the change in knee

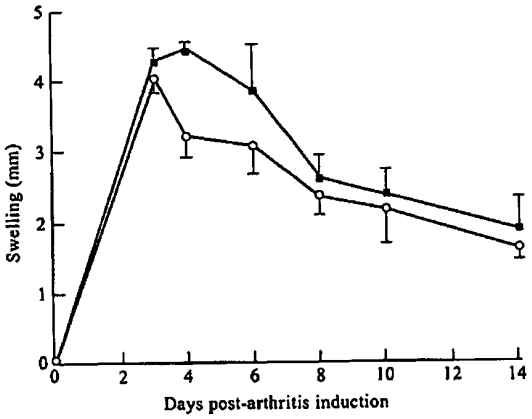


Fig. 3. Effect of treatment of established antigen arthritis with a single intra-articular dose of sCR1 given 3 days after development of disease. Results are means of five animals ± s.e.m. Significant differences in knee swelling between animals in the intra-articular sCR1-treated (○) and untreated control (■) groups was seen on day 4 (1 day after treatment) ($P<0.02$), which became non-significant thereafter. Data are from a single study. The study was repeated twice with comparable results.

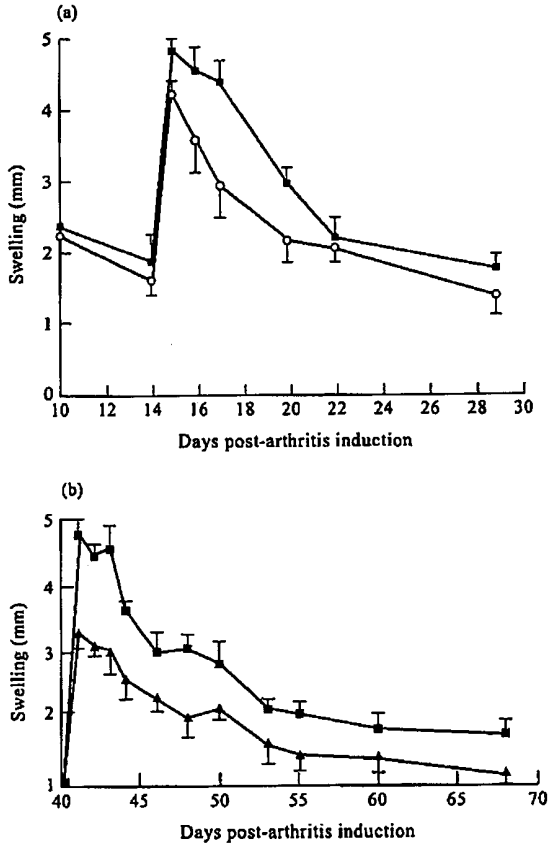


Fig. 4. Effect of a single dose of sCR1 on the development of a disease flare. (a) Flare was initiated on day 14 of already established arthritis by administration of a repeated dose of methylated bovine serum albumin (mBSA) to the right knee. Animals treated with a dose of intra-articular sCR1 at the time of flare induction (O) had significantly less knee swelling ($P<0.02$) from days 16 to 20 compared with animals that received mBSA alone (■). (b) Flare was initiated on day 40 of already established arthritis by administration of a repeated dose of mBSA to the right knee. Animals treated with a dose of intra-articular sCR1 at the time of flare induction (▲) had significantly less knee swelling ($P<0.05$) on days 42–44 compared with animals that received mBSA alone (■). Data are from a single study. The study was repeated twice with comparable results.

swelling. Comparison of the prophylactic effects of the different treatment regimens 14 days after arthritis onset revealed no statistically significant difference in the histological score between controls and those which received systemic therapy with either CVF or sCR1. However, intra-articular delivery of sCR1 improved the histological picture significantly ($P<0.0002$). The combination of both routes of therapy did not further enhance the effect of intra-articular therapy alone.

Table 1. The effect of complement inhibition upon the histological progression of antigen-induced arthritis in rats

Treatment protocol	Individual histological score
Untreated control	4, 3, 4, 4, 4, 4, 4, 3, 4
i.v. sCR1 (on day −2)	2, 2, 3, 2, 4
i.a. sCR1 (on day 0)*	0, 0, 0, 1, 0, 2, 3, 1, 0
i.v. sCR1 (on day −2) and i.a. sCR1 (on day 0)†	1, 0, 0, 1
i.p. CVF (on day −2)	4, 2, 3, 3, 2, 3, 1
i.p. CVF (on day −2) and i.a. sCR1 (on day 0)‡	1, 0, 0, 0, 1

Data are from a single study. The study was repeated three times with comparable results.

Individual histological scores, as assessed by a blinded observer, for the different treatment groups, are reported. When the scores from treated rats were compared with untreated controls by the Wilcoxon sum of ranks test:

*$P<0.002$ for intra-articular (i.a.) sCR1 administered on day 0.

†$P=0.05$ for combination therapy with sCR1 administered by i.v. injection on day −2 and i.a. sCR1 administered on day 0.

‡$P<0.002$ for combination therapy with cobra venom factor (CVF) administered by i.p. injection on day −2 and i.a. sCR1 administered on day 0.

DISCUSSION

RA is a chronic disease of unknown aetiology affecting principally synovial joints. The stimulus which maintains joint inflammation is unclear, but recent observation that the infusion of anti-tumour necrosis factor-alpha (TNF-α) MoAb suppresses joint inflammation in patients with RA highlights the importance of this macrophage-derived cytokine [19]. C-binding immune complexes or other C-derived inflammatory mediators are present within the joint and may also be important in initiating and perpetuating the inflammatory response contributing to joint damage [20–23].

The relevance of animal models to human disease has always been questioned. No animal model is strictly analogous to human RA, yet historically they have been used as proving grounds for potential therapeutic agents. Activation of the C system has been described in three recognized models of RA, namely, rat collagen-induced arthritis [24], streptococcal cell wall arthritis in rats [25], and, recently, cationic immune complex arthritis in mice [26]. It has been reported that the development of joint inflammation in each of these animal models is modified by complement depletion with CVF [24–26]. It is not, however, feasible to exploit this inhibitory effect and use this agent as a therapy in humans, as CVF is highly immunogenic and promotes the generation of proinflammatory mediators (C3a, C5a and the membrane attack complex) by activating the C pathway to completion. The development of safe, specific and potent C inhibitors such as sCR1 generated new interest in the potential manipulation of the C system in a range of inflammatory diseases.

We wished to test the effects of sCR1 in a rat model of arthritis. We chose antigen arthritis, a model in which the role of C has not previously been examined. Dumonde & Glynn [27] initially described antigen arthritis as a chronic inflammatory response in rabbits immunized with homologous and heterologous fibrin. Antigen arthritis has since been induced in rodents by immunization with a variety of antigens, such as ovalbumin [22], BSA [28] or mBSA [16], followed by the intra-articular injection of the same antigen. Antigen arthritis is a modified Arthus reaction, in which immune complexes are deposited in articular collagenous tissues, where they may activate C and other effector systems [20]. The model possesses several key similarities with human RA, notably

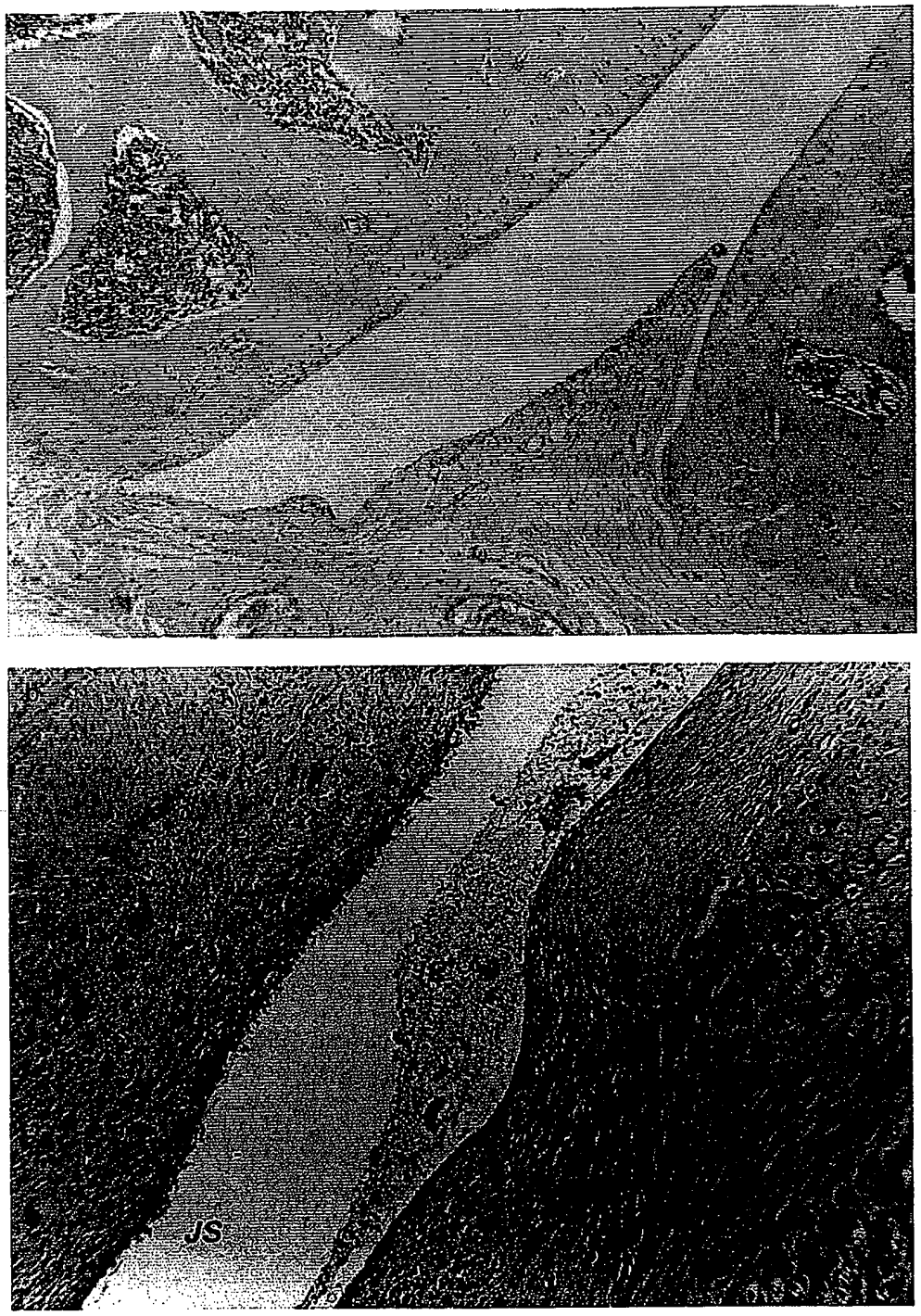


Fig. 5. (See next page.) Representative haematoxylin–eosin-stained sections of knee joints isolated from rats with antigen arthritis. (a) Intra-articular sCR1-treated rat showing no synovial infiltrate, histological score = 0. (b) Inflammatory exudate (IE) in the joint space (JS), histological score = 2.

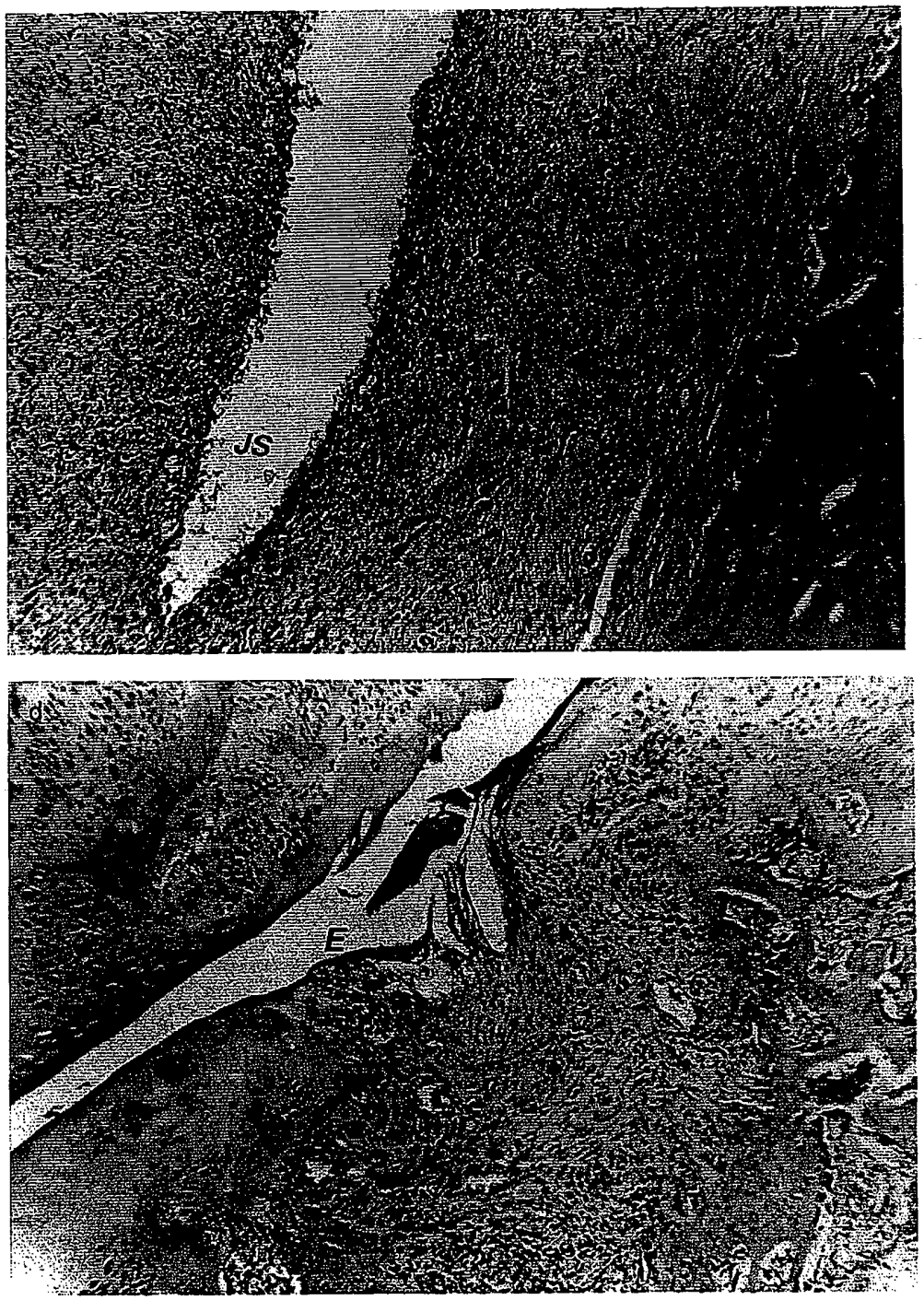


Fig. 5 (c) Severe synovial inflammatory infiltrate (SI) with no bone erosions, histological score = 3. (d) Untreated control rat showing synovial infiltrate and full thickness cartilage destruction and bone erosion (E); histological score = 4. (Mag. × 10.)

the deposition and persistence of immune complexes within the joint; the joint pathology which includes hyperplasia of synovium forming pannus and cartilage erosions, synovial biochemistry and response to anti-inflammatory drugs are also similar to those seen in human RA. We thus reasoned that this model was the best available for testing this potential therapy.

It was impossible to obtain complete inhibition of C by daily i.v. administration of sCR1, using doses and therapy schedules which have been reported by others to give complete inhibition of C in rats [15]. The reasons for this discrepancy are uncertain, but using standard assay systems we consistently found that the effects of sCR1 were short-lived (Fig. 1). Nevertheless, despite the incomplete inhibition of C we did observe a small, transient, but significant reduction in joint swelling (Fig. 2a). A recently published paper examining the effects of sCR1 on vascular injury and inflammation during renal allograft rejection in the rat showed identical kinetics of C inhibition, and despite the inability to cause sustained complete C inhibition a beneficial therapeutic effect was seen [38].

In order to eliminate completely the effects of systemic C, we used CVF, a single i.p. dose of which caused complete C inhibition for >3 days. The effects on joint inflammation were no greater than with the partial C inhibition caused by sCR1. Neutrophil accumulation within the joints was similar to that in untreated animals with both methods of inhibiting systemic C. This suggested either that C played a minor role in this model, or that C biosynthesis in the joint was involved. sCR1 is a large molecule with low tissue penetrance; it is thus unlikely to enter the joint in significant amounts. Therefore in order to explore the role of intra-articular C synthesis we examined the effects of intra-articular administration of sCR1 on the development of disease. Intra-articular therapy reduced joint swelling to a far greater degree than systemic decomplementation alone (Fig. 2b), whereas the combination of both i.v. and intra-articular routes did not have an additive effect. Importantly, intra-articular treatment with sCR1 also markedly inhibited inflammation and destructive changes in the joint (Table 1 and Fig. 5). The effects of intra-articular therapy suggest that the synovial space has the potential to behave as an isolated environment capable of producing C components, and that local C biosynthesis and activation is a driving force behind the inflammatory response. Such a scenario has been demonstrated in other tissue sites, e.g. kidney [29], brain [30], and suggested to occur in the joint [31]. Indeed, it has been proposed that local C synthesis may be more relevant to disease in numerous situations [32].

In normal and arthritic synovium there is a variety of cell types which are capable of producing C components. Infiltrating polymorphonuclear cells are capable of producing C3, C6, C7 [33], activated macrophages C1, C4, C2, C3, C5 [34], and resident synovial fibroblasts C3 and several other components [35–37]. As these cells are all present in the inflammatory pannus and at the sites of bony erosions, the local synthesis of C could contribute significantly to inflammation. In this context it is worth noting that a study of C3 metabolism in a patient with RA showed that approximately half the C3 present in the joint had been synthesized locally [38]. It has recently been shown that inhibition of the membrane regulator, CD59, in the normal rat knee joint triggers an acute transient inflammatory arthritis [31]. The membrane attack complex is deposited on the synovial cells even after systemic decomplementation, suggesting that the synovium is the source of C in this model, thus providing further evidence for the importance of local C biosynthesis in initiating joint inflammation.

Having demonstrated that C has a role to play in the induction of disease in this model, we proceeded to examine its role in established disease, as this more closely mimics the potential therapeutic option in human arthritis. Different results were seen depending on the timing of treatment, treating 3 days after onset of disease causing a significant, albeit small and transient, reduction in inflammation, whereas treating 7 days after disease onset did not influence the established inflammation. This suggests that different components of the immune system have different relative contributions to the persisting inflammation at different stages of disease. This supports the findings of Griffiths [16] in this disease model, who reported that different cellular populations predominate at different stages of disease, neutrophils predominating during the early stages, whereas macrophages and T cells were more abundant during the chronic phase.

Rheumatoid disease is a chronic disease characterized by recurrent exacerbations of joint inflammation. Unlike other models of arthritis, e.g. collagen-induced arthritis [24], the antigen-induced arthritis model offers the possibility to induce similar flares of disease by repeated application of the intra-articular antigen. Flare was induced 14 and 40 days after initial disease onset. The pathology at the time of flare induction is characterized by macrophages and T cells populating the joint in increasing numbers in contrast to the abundance of neutrophils in the acute disease [16]. Treating with intra-articular sCR1 had a small but significant beneficial effect on the swelling which persisted for 3 days. It did not, however, influence the ultimate course of the disease. The data suggest that during a flare of disease or repeat intra-articular challenge there is increased generation of immune complexes which trigger C activation, with the consequent recruitment of neutrophils and production of inflammatory mediators. Inhibition of C thus dampens the flare but has no long-term effect on the underlying chronic disease.

In conclusion, local inhibition of C with a single dose of intra-articular sCR1 had a beneficial effect on the course of antigen arthritis when given at the time of disease induction or during the induction of a flare of established disease. The effects observed on established disease and disease flares were modest and transient. However, sCR1 is a human C inhibitor, the efficacy of which is much reduced in rats [10]. It is possible that its more powerful inhibitory effect on human C and longer half-life in man will make it a much better inhibitor of human disease. The intra-articular route of administration is an attractive prospect, as the requirement of C for host defence against infection may preclude prolonged systemic inhibition of C as a therapeutic strategy. Local inhibition caused no reduction in systemic C activity, and offered additional therapeutic advantages as the doses required for local treatment were much lower than those necessary for systemic treatment. We suggest that local therapy with sCR1 offers an exciting prospect for treatment of human rheumatic disease.

ACKNOWLEDGMENTS

We thank T Cell Sciences Inc. for the supply of sCR1 (TP10) and the ARC for their financial support during the course of this study (ARC Clinical Fellowship Award to R.M.G., reference no. G0507).

REFERENCES

1 Morgan BP. The biological effects of complement activation. In: Complement, clinical aspects and relevance to disease. London: Academic Press, 1990:36–56.

2 Zvaifler NJ. Breakdown products of C3 in human synovial fluids. J Clin Invest 1969; 48:1532–42.
3 Ward PA, Zvaifler NJ. Complement derived leukotactic factors in inflammatory synovial fluids of humans. J Clin Invest 1971; 50:606–16.
4 Morgan BP, Daniels RH, Williams BD. Measurement of terminal complement complexes in rheumatoid arthritis. Clin Exp Immunol 1988; 73:474–8.
5 Brodeur JP, Ruddy S, Schwartz LB, Moxley G. Synovial fluid levels of complement SC5b-9 and fragments Bb are elevated in patients with rheumatoid arthritis. Arthritis Rheum 1991; 34:1531–7.
6 Corvetta A, Pomonio G, Rinaldi N, Luchietti MM, Diloretto C. Terminal complement complex in synovial tissue from patients affected by rheumatoid arthritis, osteoarthritis and acute joint trauma. Clin Exp Rheumatol 1992; 10:433–8.
7 Hogasen K, Mollnes TE, Harboe M, Gotze O, Hammer HB, Opperman M. Terminal complement activation and low lysis inhibitors in rheumatoid arthritis synovial fluid. J Rheumatol 1995; 22:24–28.
8 Daniels RH, Houston WAJ, Petersen MM, Williams JD, Williams BD, Morgan BP. Stimulation of human rheumatoid synovial cells by non-lethal complement membrane attack. Immunology 1990; 69:237–42.
9 Daniels RH, Williams BD, Morgan BP. Human rheumatoid synovial cell stimulation by the membrane attack complex and other pore-forming toxins *in vitro*: the role of calcium in cell activation. Immunology 1990; 71:312–6.
10 Weisman MF, Barlow T, Leppo MK *et al.* Soluble human complement receptor type 1. *In vivo* inhibitor of complement suppressing post ischaemic myocardial inflammation and necrosis. Science 1990; 249:146–51.
11 Homeister JW, Satoh PS, Kilgove KS, Lucchesi BR. Soluble complement receptor type 1 prevents human complement mediated damage of the rabbit isolated heart. J Immunol 1993; 150:1055–64.
12 Hill J, Lindsay TF, Ortiz F, Teh; CG, Mechtman MB, Moore FD. Soluble complement receptor type 1 ameliorates the local and remote organ injury after intestinal ischaemia reperfusion in the rat. J Immunol 1992; 149:1723–8.
13 Zehr KJ, Merskowitz A, Lee PC, Kumar P, Culllinor AM, Baumgartner WA. Neutrophil adhesion and complement inhibition prolongs survival of cardiac xenografts in discordant species. Transplantation 1994; 57:900–6.
14 Mulligan MS, Teh GC, Rudolph AR, Ward PA. Protective effect of soluble CR1 in complement and neutrophil mediated tissue injury. J Immunol 1992; 48:1479–85.
15 Piddlesden SJ, Storch MK, Hobbs M, Freeman AM, Larsmann M, Morgan BP. Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody mediated demyelinating experimental allergic encephalomyelitis (ADEAE). J Immunol 1994; 152:5477–84.
16 Griffiths RJ. Characterisation and pharmacological sensitivity of antigen arthritis is induced by methylated bovine serum albumin in the rat. Agents Actions 1992; 35:88–95.
17 Vogel CW, Muller-Eberhard J. Cobra venom factor : an improved method for purification and biochemical characterisation. J Immunol Methods 1984; 73:203–20.
18 Whaley K. Measurement of complement. In: Whaley K, ed. Methods in complement for clinical immunologists. Edinburgh: Churchill Livingstone 1985:77–139.
19 Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Bijl H, Woody JN. Repeated therapy with monoclonal antibodies to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. Lancet 1994; 344:1125–7.
20 Jasin HE, Cooke TD. The inflammatory role of immune complexes trapped in joint collagenous tissues. Clin Exp Immunol 1978; 33: 416–24.
21 Cooke D, Hurd E, Ziff M, Jasin H. The pathogenesis of chronic inflammation in experimental antigen-induced arthritis 11. Preferential localisation of antigen–antibody complexes to collagenous tissues. J Exp Med 1972; 135:323–37.
22 Brauer R, Thoms S, Henzgen S, Waldman G. Significance of cell mediated and humoral immunity in the acute and chronic phase of antigen induced arthritis in rabbits. Exp Pathol 1988; 34:197–208.
23 Jasin HE. Mechanism of trapping immune complexes in joint collagenous tissue. Clin Exp Immunol 1975; 22:473–85.
24 Morgan K, Clague RB, Shaw MJ, Firth SA, Twose TM, Lennox Holt PJ. Native type 2 collagen induced arthritis in the rat. The effect of complement depletion by cobra venom factor. Arthritis Rheum 1981; 24:1356–62.
25 Schwab JH, Allen JB, Anderle SK, Dallorf F, Eisenberg R, Cromartie WJ. Relationship of complement to experimental arthritis induced in rats by streptococcal cell walls. Immunology 1982; 46:83–87.
26 van Lent PLEM, van den Bersselaar LAM, van den Hoek AEM, van de Loo AAJ, van den Berg WB. Cationic immune complex arthritis in mice—a new model. synergistic effect of complement and interleukin 1. Am J Pathol 1992; 49:1451–61.
27 Dumonde DC, Glynn LE. The production of arthritis in rabbits by an immunological reaction to fibrin. Br J Exp Pathol 1962; 43:373–83.
28 Brackertz D, Mitchell G, Mackay IR. Antigen induced arthritis in mice 1. Induction of arthritis in various strains of mice. Arthritis Rheum 1977; 20:841–50.
29 Sacks S, Zhou W, Campbell RD, Martin J. C3 and C4 gene expression and gamma interferon mediated regulation in human glomerular mesangial cells. Clin Exp Immunol 1993; 3:411–7.
30 Gasque P, Fontaine M, Morgan BP. Complement expression in the brain: biosynthesis of terminal pathway components and regulators in human glial cells and cell lines. J Immunol 1995; 154:4726–33.
31 Mizuno M, Nishsikawa K, Goodfellow RM, Piddlesden SJ, Morgan BP, Matsuo S. The effects of functional suppression of a membrane bound complement regulatory protein CD59 in the synovial tissue in rats. Arthritis Rheum 1997; 40:527–33.
32 Morgan BP, Gasque P. Extrahepatic complement biosynthesis: where when and why? Clin Exp Immunol 1997; 107:1–7.
33 Hogasen A, Wurzner R, Abrahamsen TG, Dierich MP. Human polymorphonuclear leucocytes store large amounts of terminal complement components C7/C6 which may be released on stimulation. J Immunol 1995; 154:4734–40.
34 DeCeulaer C, Papakogolou S, Whaley K. Increased biosynthesis of complement components of cultured monocytes, synovial macrophages and synovial membrane cells from patients with rheumatoid arthritis. Immunology 1980; 41:37–43.
35 Colten HR, Struck RC. Synthesis of complement components at extra hepatic sites. In: Whaley K, Loos M, Weiler J, eds. Complement in health and disease, 2nd edn. Lancaster: Kluwer Academic, 1993:127–58.
36 Guc D, Gulati P, Lemercier C, Lappin D, Bimie GD, Whaley K. Expression of the components and regulatory proteins of the alternative complement pathway and the membrane attack complex in normal and diseased synovium. Rheumatol Int 1993; 13:139–46.
37 Ruddy S, Colten HR. Biosynthesis of complement proteins by synovial tissue. N Engl J Med 1974; 290:1284–8.
38 Pratt JR, Hibbs MJ, Laver AJ, Smith RAG, Sacks SH. Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat. Am J Pathol 1996; 149:2055–66.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-6

This is Exhibit SMT-6 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Joni Russ

JUSTICE OF THE PEACE (QUALIFIED)
No. 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

Antigen-Induced Arthritis Model

Introduction:

Antigen-induced arthritis, first described in 1962, is an established model of RA that involves stimulation of T-lymphocyte reactivity against the immunising antigen (Dumonde and Glynn, 1962; Griffiths, 1992). This model is induced by the immunisation of animals with a protein antigen (mBSA, ovalbumin or fibrin) and an adjuvant, followed by the intra-articular injection of the same antigen (Dumonde and Glynn, 1962; Brackertz *et al.*, 1977; Yoshino, 1996; Simon *et al.*, 2001). This results in an immune-complex mediated inflammatory response, characterised by chronic synovitis, which is localised to the injected joint. The ability to localise inflammation to the antigen-injected joint only (monoarticular arthritis) allows for an internal control in the contra-lateral joint (Griffiths, 1992). Many of the disease pathologies in this model mimic those seen in human RA (Table 1), having both acute and chronic phases of disease (Griffiths, 1992). There is also the capacity to induce subsequent flare-ups (as seen in RA), through the re-injection of the antigen (Griffiths, 1992; Goodfellow *et al.*, 1997).

Table 1: Animal models of rheumatoid arthritis: relevance to human condition.

Disease Model	Similarities to human disease
Antigen-Induced (Monoarticular) Arthritis	- Infiltration of PMNs, macrophages and T-lymphocytes - Swelling of affected joint - Hyperplasia of synovial membrane and synovitis - Cytokine involvement in various stages of disease - Tissue remodeling - Similar histology: cartilage erosions and pannus formation - Induction of flare-up reaction (local T-cell hyperreactivity) - Immune-complex mediated - Complement involvement - Drug responsiveness
Adjuvant Arthritis	- Polyarthropathy of the small peripheral joints and edema - Unknown autoantigen - Osteoclast involvement - Tissue remodeling and fibrosis - Similar histology: bone erosions and pannus formation - Complement involvement - Drug responsiveness
Collagen-Induced Arthritis	- Polyarthropathy of the small peripheral joints - Synovitis and periarticular inflammation - Infiltration of PMNs and mononuclear cells - Significant cytokine involvement - Tissue remodeling - Similar histology: bone and cartilage erosions, and pannus formation - Specific MHC association - Immune-complex deposition within joint - Complement involvement - Drug responsiveness

Information from Johnson *et al.*, 1986; Griffiths, 1992; Griffiths *et al.*, 1992; Griffiths *et al.*, 1995; Yoshino, 1996; Goodfellow *et al.*, 1997; Myers *et al.*, 1997; Williams, 1998; Bendele *et al.*, 1999; Joe *et al.*, 1999; Linton & Morgan, 1999; Goodfellow *et al.*, 2000; van den Berg, 2000; Mizuno *et al.*, 2000.

Methods:

Female Wistar rats (225-275g) were obtained from the CABH (Pinjarra Hills, The University of Queensland). Methylated bovine serum albumin (mBSA; Sigma, USA) was suspended in Freund's complete adjuvant (Sigma, USA) at the concentration of 1 mg/mL and sonicated for 10 min to ensure homogeneity. Rats were sensitised to the mBSA by the subcutaneous injection of 0.5 mL of this solution into the hind flanks 2 and 3 weeks prior to challenge (Days –21 and –14). Two weeks after the second injection (Day 0), rats were anaesthetised with ketamine (80 mg/kg i.p.) and xylazine (12 mg/kg i.p.) and both hind legs shaved. A homogenous suspension of 0.5 mg mBSA in 100 μL saline was aseptically injected into the joint space of the right knee, with the contra-lateral left knee receiving 100 μL saline alone. Sham-operated rats received 100 μL saline in both the left and right knees. Rats were then allowed to recover after being placed in separate cages. In the first study, rats were examined for 14 or 28 days after mBSA injection (Day +14 or +28) before being euthanased under anaesthesia prior to sample collection. In the second study, on Day 28 rats were re-anaesthetised with ketamine (80 mg/kg i.p.) and xylazine (12 mg/kg i.p.) and 0.5 mg mBSA in 100 μL saline was again aseptically injected into right knee joint space, with the contra-lateral left knee receiving 100 μL saline. These rats were examined for a further 21 days (Day +49) before sample collection following euthanasia under anaesthesia.

The body weight of rats in the 28-day study was measured periodically throughout the experiment. Results are expressed as the change in body weight following mBSA challenge on Day 0.

Right and left knee swelling were quantitatively assessed at various time points during the study period by measuring the medial-lateral width across the joint of

constrained rats with vernier calipers. Results are expressed as the change in left or right knee swelling following mBSA challenge on Day 0, or as a change from Day 4 for rats treated from Day 4-28 with the C5a receptor antagonist.

The appearance of each rat's gait was also scored during the study on a scale of 0-4 as described below.

Score	Description
0	No limp, full weight-bearing on hind right leg.
1	Mild limp.
2	Moderate limp.
3	Severe limp.
4	No weight-bearing on hind right leg.

At the completion of the 14 and 28-day study, the left and right knee joints of rats were lavaged with 100 μL saline. The number of total cells in this lavage fluid was then determined using a haemocytometer. Differential cell counts were then performed on air-dried glass smears of lavage samples stained with Wright's stain (Sigma, USA). Red blood cells were excluded from the cell counts and the total number of macrophages, PMNs and lymphocytes was expressed as the number of cells/mL lavage fluid.

Levels of TNF-α were measured in the serum and intra-articular knee lavage samples collected from rats at the completion of the study. An enzyme-linked immunoabsorbant assay (ELISA) antibody set (OptEIA, Pharmingen, USA) was used according to manufacturer's instructions to measure these TNF-α levels. A 96-well round-bottomed immunosorbant plate (Nunc Maxisorb, USA) was coated with 100 μL rabbit anti-rat TNF-α antibody (capture antibody) diluted 1:200 with coating buffer (0.1 M carbonate,

pH 9.5) and incubated overnight at 4°C. The plate was then washed 3 times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween) and 200 μL of PBS with 10% fetal calf serum added to each well. Following a 1 hour incubation period, the plate was washed 5 times with PBS-Tween and 100 μL samples added in duplicate. Recombinant rat TNF-α was diluted from 4000 ng/ml to 30 pg/ml and added in duplicate to each plate to serve as a standard curve. The plate was then incubated for a further 2 hours at 37°C followed by 5 washes in PBS-Tween. Diluted biotinylated anti-rat TNF-α monoclonal antibody (detection antibody, 100μL) was then added to each well and the plate incubated at RT for 1 hour. The plate was then washed 5 times with PBS-Tween and 100 μL of avidin-horseradish peroxidase conjugate (enzyme reagent) added to each well. After a 30 min incubation period at RT, the plate was washed 7 times with PBS-Tween and 100 μL of substrate (tetramethylbenzidine) added to each well. Colour was allowed to develop in the dark over 30 min followed by addition of 50 μL of 0.5 M H_2SO_4 (stop solution). Absorbance was read at 450 nm and concentrations of TNF-α determined by linear regression analysis from the standard curve. Serum and intra-articular fluids obtained as described in previous sections were stored at -20°C and samples were analyzed within 2 weeks of collection. Results are expressed as ng/g tissue.

After the completion of each study and following the lavaging of knees, the knee joints from every rat were dissected out and then stored in 10% buffered formalin for at least 10 days. Knee samples were then decalcified in a saturated solution of ethylenediaminetetraacetic acid (EDTA) for 21 days. They were then embedded in paraffin wax and sections were cut, mounted and stained using a haematoxylin and eosin stain. Each

section was then scored by Dr. Ian Shiels in a blinded fashion and scored for the level of joint damage in a scale from 0-4 as described below.

Score	Description
0	No abnormalities.
1	Mild inflammatory cell infiltration in the synovial membrane with no significant thickening of the membrane or cartilage erosion.
2	Extensive inflammatory cell infiltration and mild thickening of the synovial membrane.
3	Extensive inflammatory cell infiltration and synovitis, thickening and fibrosis of the joint capsule and cartilage involvement without erosions.
4	Extensive inflammatory cell infiltration and synovitis, significant thickening and fibrosis of the joint capsule and involvement of the articular cartilage with erosions.

Representative histological sections of each of these scores are shown in Figure 2.1 under 40 x magnification.

A 14- and 28-day (single antigen injection) or 49-day (two antigen injections) study period were used to examine the therapeutic effects of the C5a antagonist on mBSA-induced monoarticular arthritis. The following groups of rats were used in the 14-day study: sham-operated (baseline controls), untreated (arthritis controls), C5a antagonist-pre-treated (1 mg/kg/day), C5a antagonist-post-treated (1 mg/kg/day), and ibuprofen-post-treated (30 mg/kg/day). The following groups of rats were used in the 28-day study: sham-operated (baseline controls), untreated (arthritis controls), C5a antagonist-pre-treated (1 and 0.3 mg/kg/day), ibuprofen-pre-treated (30 mg/kg/day), C5a antagonist (1 mg/kg/day) and

ibuprofen (30 mg/kg/day)-pre-treated combination, and C5a antagonist-post-treated (3 mg/kg/day). Rats that were pre-treated received drug treatment 2 days prior to arthritis induction (Day –2) and then daily throughout the study. Rats that were post-treated received drug treatment 4 days after the induction of arthritis (Day +4) and then daily throughout the study. In the 49-day study, a further 2 groups were examined: untreated rats, and C5a antagonist-pre-treated rats (1 mg/kg/day). In both studies, various parameters were measured in each group at regular intervals over the course of the study.

Results:

14-day study. Measurements of the saline-injected left knee of each rat did not significantly change from pre-injection values during the course of each experiment (data not shown). Following the injection of mBSA at Day 0 in drug-free sensitized rats, the average increase in the width of the right knee peaked at Day +3 (+4.69 ± 0.32 mm, n = 11; Figure 1A). Rats which had been pretreated from Day –2 with AcF-[OPdChaWR] (1 mg/kg/day), had significantly reduced right knee widths from Days +2-14 (peak at Day +3: +2.08 ± 0.59 mm, n = 9; $P < 0.05$) compared to drug-free arthritic knees (Figure 1A). Gait scores in drug-free arthritic rats also increased above baseline levels following induction of arthritis (Figure 1B). Pretreatment with the C5a antagonist significantly decreased these scores from Days +2-14 ($P < 0.05$). There was a high correlation between gait scores and knee swelling in drug-free arthritic rats for all 3 experimental trials (14 day trial: $r^2 = 0.83$, n = 7; 28 day trial: $r^2 = 0.96$, n = 11; 49 day trial: $r^2 = 0.84$, n = 17) for Days +2 to completion of study.

In a separate study, the effects of the C5a antagonist or ibuprofen on knee swelling and gait scores following the establishment of arthritis were examined. Rats treated with either AcF-[OPdChaWR] (1 mg/kg/day) or ibuprofen (30 mg/kg/day) from Days +2-14, had significantly reduced knee swelling (Days +3-14) and gait scores (Days +4-14) compared to arthritic rats which received no drug treatment ($P < 0.05$; Figure 1C/D).

In the initial 14-day study, TNFα and IL-6 levels were found to be elevated in the right knee lavage fluid on Day 14, as were TNFα levels in the serum of drug-free arthritic rats (Figure 2A/B). Rats pretreated with the C5a antagonist had significantly lower levels of these cytokines ($P < 0.05$; Figure 2A/B) in the joint and serum at Day 14. The majority (>90%) of cells recovered from the right knee lavage fluid at Day 14 were PMNs. Significantly fewer PMNs were found in the lavage fluid of rats treated with AcF-[OPdChaWR] or ibuprofen throughout the study, or in rats treated with the C5a antagonist from Day +2, compared to drug-free arthritic rats ($P < 0.05$; Figure 2C).

The saline-injected left knees of all rats in every study showed no histological abnormalities (Figure 3A) and all were scored 0 (data not shown). Sections from the right knees of drug-free arthritic rats on Day 14 had marked cellular infiltration, which were predominantly neutrophils, and mild synovial cell proliferation, with an average histopathology score of 3.2 ± 0.3 ($n = 16$, Figure 2D). Histological sections from rats pretreated with AcF-[OPdChaWR] had a lesser degree of cellular infiltration and synovial proliferation, resulting in a significantly lower histopathological score of 1.4 ± 0.5 ($n = 8$; $P < 0.05$; Figures 2D). Histological sections from rats post-treated with the C5a antagonist, 2 days after the induction of arthritis, also had a lower degree of cellular infiltration and synovial proliferation compared to drug-free arthritic rats, although to a lesser extent than

rats pretreated at Day –2 with the C5a antagonist. The mean histopathological scores from these post-treated rats was 2.2 ± 0.4 which was significantly lower than drug-free arthritic rats (n = 8; $P < 0.05$; Figure 2D). In contrast, histological sections from rats treated with ibuprofen (30 mg/kg/day) from Days +2-14, had no change in histological parameters compared to drug-free arthritic rats (score of 3.5 ± 0.3; n = 6; Figures 2D).

28-day study. Following arthritis induction, right knee widths of drug-free arthritic rats rapidly increased above baseline to peak at Day +3 (+4.98 ± 0.39 mm above baseline; n = 14) and slowly decreased to +0.51 ± 0.21 mm at Day 28 (Figure 3A). Rats pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from Day –2, had significantly lower right knee widths from Days +1-28 (peak at Day +3: +2.66 ± 0.52 mm, n = 12; $P < 0.05$; Figure 3A). Rats pretreated with AcF-[OPdChaWR] at 0.3 mg/kg/day, also had significantly lower right knee widths from Days +2-15 ($P < 0.05$; Figure 3A). Rats pretreated with ibuprofen (30 mg/kg/day) had significantly decreased knee widths from Days +2-8 only (peak at Day +3: +2.30 ± 0.30 mm; n = 11; $P < 0.05$; Figure 3A). Rats receiving a combination of the C5a antagonist (1 mg/kg/day) and ibuprofen (30 mg/kg/day) from Day –2, had a significant reduction in right knee widths from Days +1-28 (peak at Day +3: +2.19 ± 0.06mm, n = 12; $P < 0.05$; Figure 3A), which was comparable to rats receiving the C5a antagonist alone. Gait scores in rats pretreated with either the C5a antagonist, ibuprofen or the combination of both were significantly reduced compared to drug-free arthritic rats from Days +1-12 ($P < 0.05$; Figure 3B).

In the 14-day studies, which were performed first in the series of studies reported here, the C5a antagonist was administered at 1 mg/kg/day both as a preventative (Days –2-14) and reversal (Days +2-14) therapy. It was noted that rats treated with the antagonist at

this dose from Day +2 had less improvement in histopathological scores compared to rats treated from Day –2 (Figure 2D). In the following 28-day reversal studies, a higher dose of C5a antagonist (3 mg/kg/day) was used to determine if this might be a more effective reversal dose regimen. It was found that rats that were treated with this dosage regime had significantly improved right knee widths (Days +5-28) and gait scores (Days +5-12) compared to drug-free arthritic rats ($P < 0.05$; Figure 3C/D).

In all rats in the 28-day studies, there were no detectable levels of TNFα, either in the serum or in the knee lavage fluid, at Day 28. The vast majority (>95%) of cells recovered in the lavage fluid of the right knees of rats were macrophages. Drug-free arthritic rats had an average of 46.5 ± 12.3 x 104 macrophages/mL lavage fluid (n = 10; Figure 5A), an ~20-fold increase over saline-injected left knees. Rats pretreated with AcF-[OPdChaWR], ibuprofen or a combination of both, or rats treated from Day +4 with the C5a antagonist alone, had significantly lower numbers of macrophages in the right knee lavage fluid (Figure 4A).

Right knee histology from arthritic drug-free rats had varying degrees of synovial cell proliferation and cellular infiltration, and these were scored overall with an average of 2.9 ± 0.3 (n = 16; Figure 4B). Sections from rats either pretreated with C5a antagonist (1 mg/kg/day) or treated from Day +4 (3 mg/kg/day), had an equal reduction in severity of lesions compared to drug-free arthritic rats, with significantly decreased scores of 1.0 ± 0.4 and 1.1 ± 0.5, respectively (n = 12 in each group; $P < 0.05$; Figure 4B). Conversely, rats pretreated with ibuprofen (30 mg/kg/day) had no improvement of right knee pathology, with an average score of 2.9 ± 0.3 (n = 6; Figure 4B). Rats pretreated with a combination of the C5a antagonist and ibuprofen also had a significant reduction in histopathological

scores, which was similar to that of rats treated with only the C5a antagonist (n = 12; Figure 4B).

49-day study. In drug-free rats following the first injection of mBSA at Day 0, the right knee increased in width with a peak swelling above baseline of +5.27 ± 0.56 mm (n = 6) on Day +3. Following the second injection of mBSA on Day 28, the increase in the width of the right knee was similar in magnitude to the first challenge, with a peak of +4.47 ± 0.60 mm above pre-injection values, 1 day after the second injection (Figure 5A). Rats pretreated with AcF-[OPdChaWR] at 1 mg/kg/day had significantly lower right knee widths compared to drug-free rats, from Days +1-49 with peaks of +2.87 ± 0.51 (Day 3) and +2.64 ± 0.37 mm (Day 29) for the first and second injection, respectively (n = 6; $P < 0.05$; Figure 5A). Gait scores increased in drug-free arthritic rats with the deterioration in gait proportional to right knee widths, with maximum gait scores following both the first and second injection (Figure 5B). C5a antagonist-dosed rats had significantly improved gait scores from Days +2-16 and +30-44 ($P < 0.05$; Figure 5B).

Examination of right knee sections from 49-day arthritic drug-free rats showed a more severe pathology than was seen in the other studies involving single intra-articular injection of antigen and more limited experimental time spans. All antigen-injected right knees in drug-free rats had marked inflammatory cell infiltration and severe synovial proliferation and fibrosis. Additionally, cartilage erosion was observed in all sections from challenged right knees, resulting in the average histopathological score of 3.9 ± 0.1 (n = 6, Figure 5C). Sections of right knees from rats which had been pretreated with AcF-[OPdChaWR] (1 mg/kg/day) from Days –2 onwards, had decreased pathology compared to drug-free arthritic rats, with a significantly improved score of 1.7 ± 0.7 (n = 6; Figure 5C).

FIGURE 1.

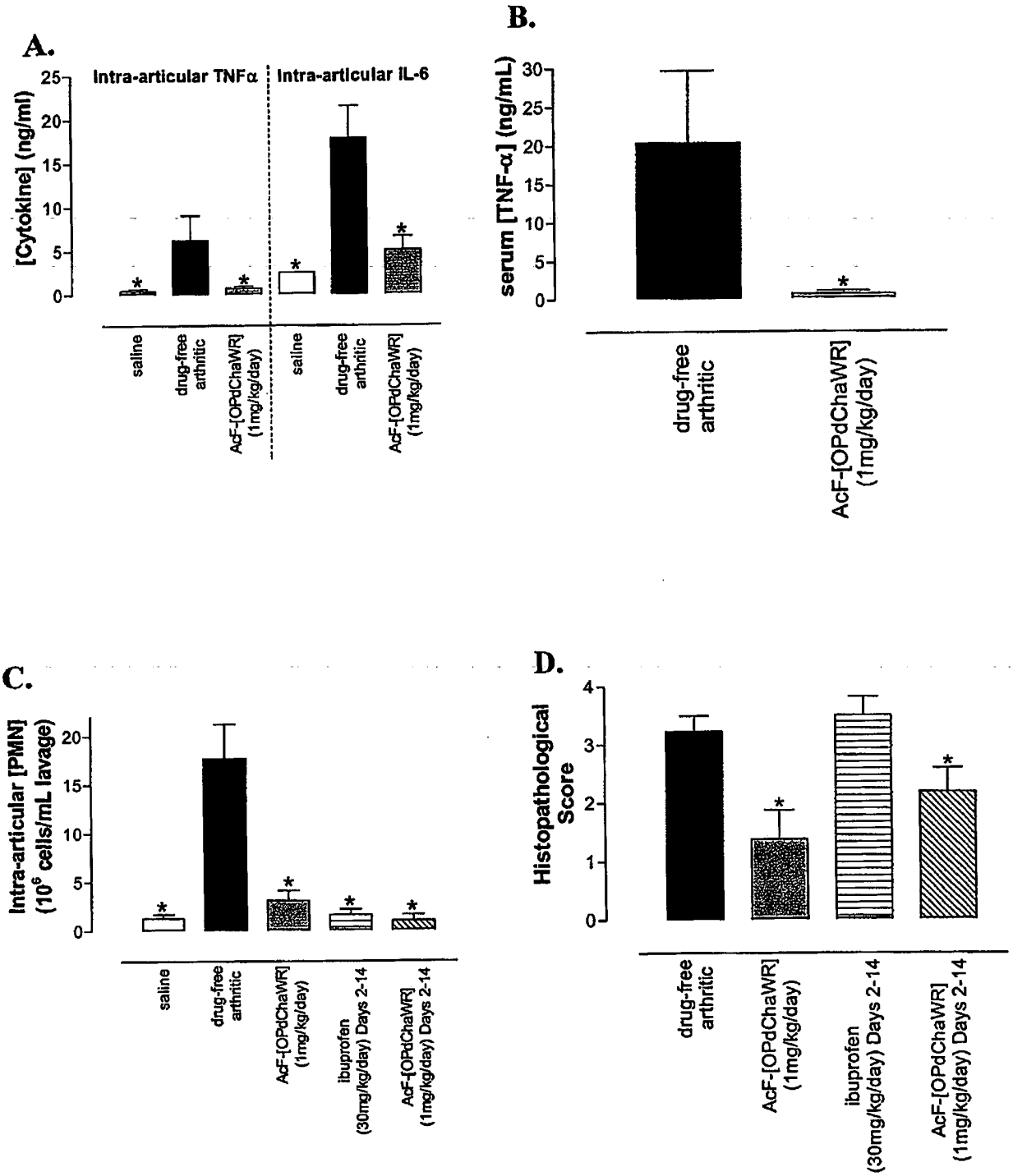

A.
Intra-articular TNFα
Intra-articular IL-6
[Cytokine] (ng/ml)
25
20
15
10
5
0
saline
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
saline
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
B.
serum [TNF-α] (ng/mL)
30
25
20
15
10
5
0
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
C.
Intra-articular [PMN] (10^6 cells/mL lavage)
20
15
10
5
0
saline
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day) Days 2-14
AcF-[OPdChaWR] (1mg/kg/day) Days 2-14
D.
Histopathological Score
4
3
2
1
0
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day) Days 2-14
AcF-[OPdChaWR] (1mg/kg/day) Days 2-14

FIGURE 2

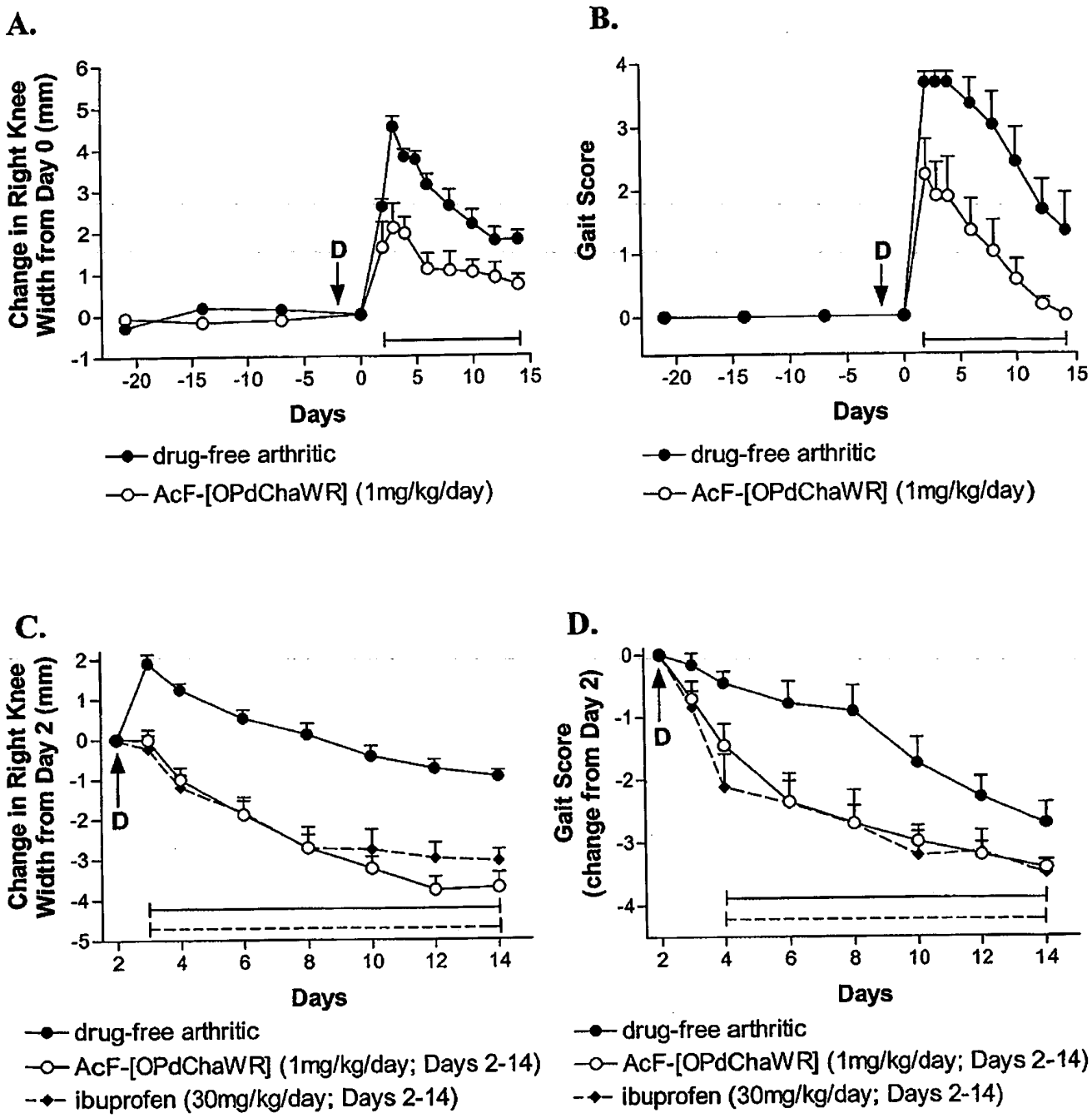

A.
Change in Right Knee Width from Day 0 (mm)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
B.
Gait Score
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
C.
Change in Right Knee Width from Day 2 (mm)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day; Days 2-14)
ibuprofen (30mg/kg/day; Days 2-14)
D.
Gait Score (change from Day 2)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day; Days 2-14)
ibuprofen (30mg/kg/day; Days 2-14)

FIGURE 3.

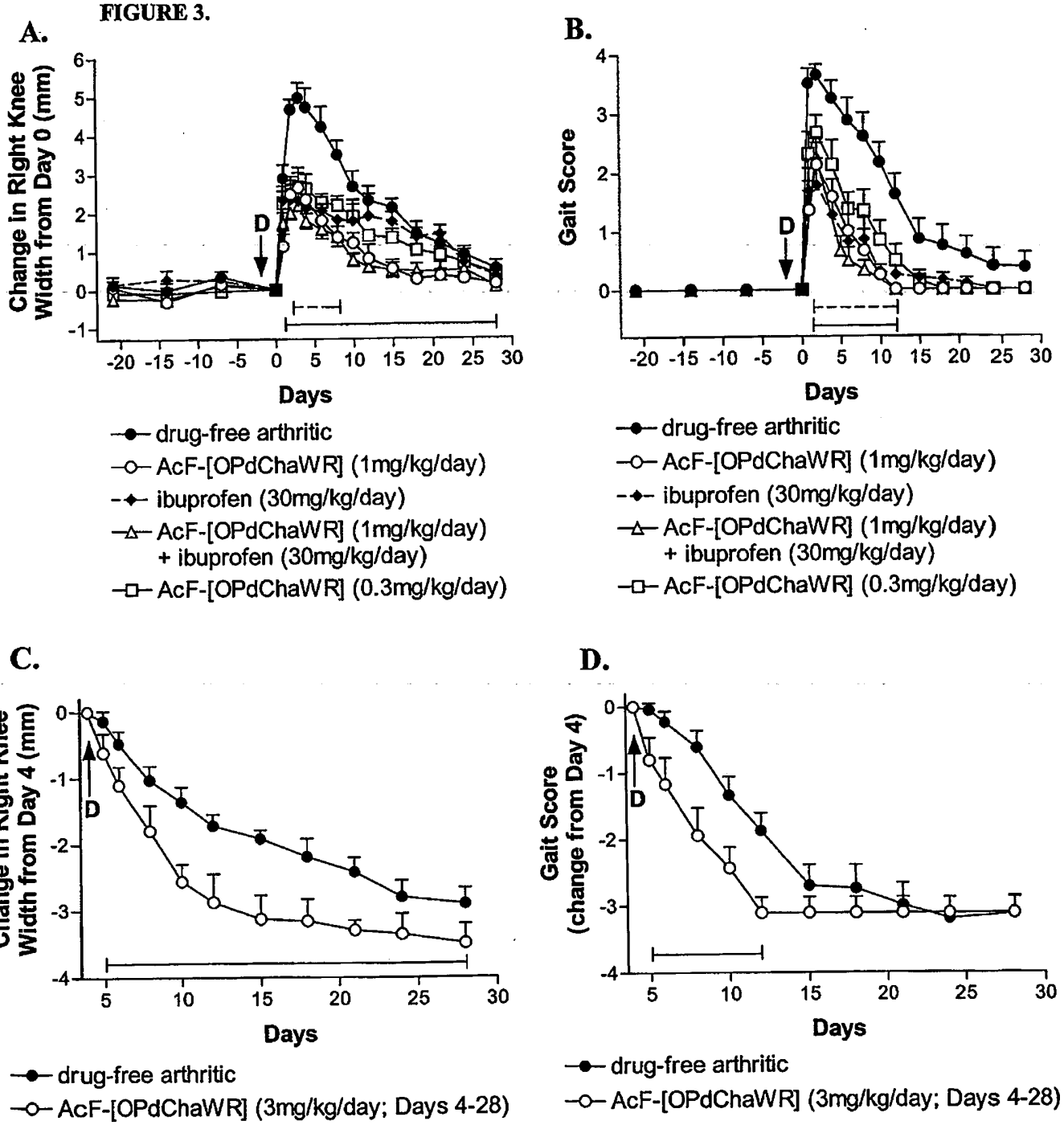
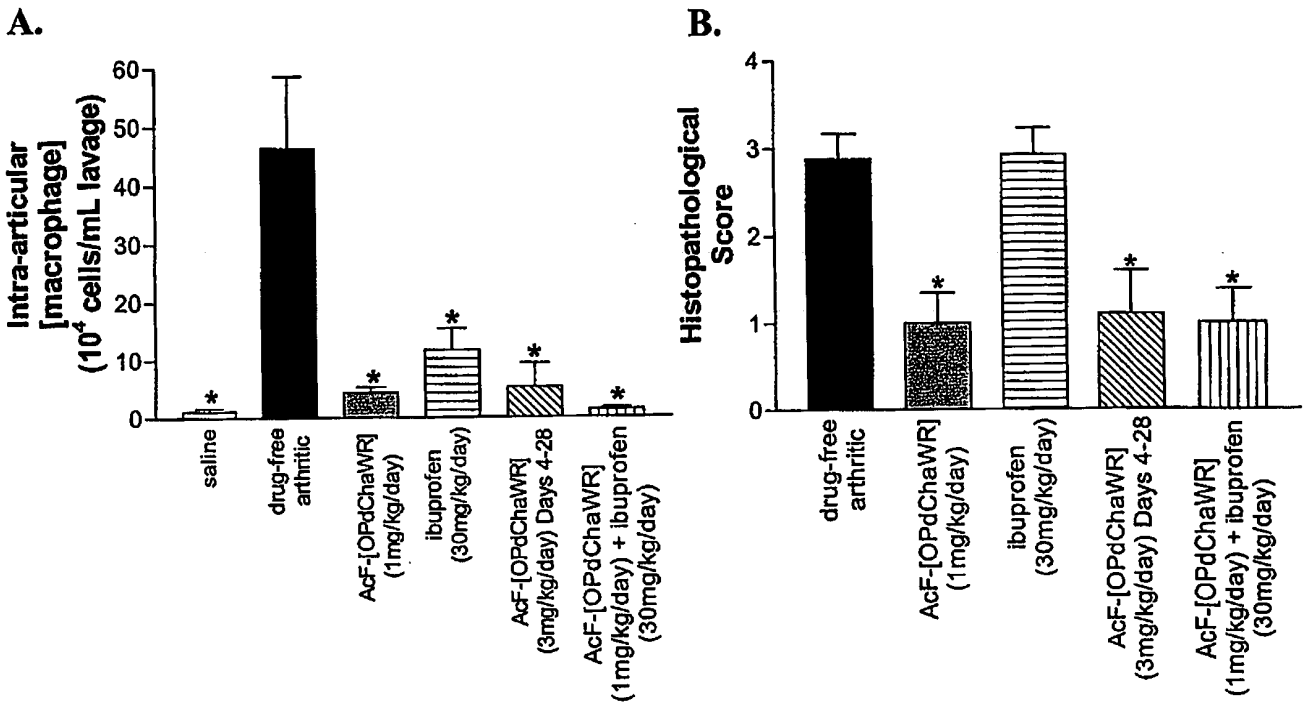

A.
Change in Right Knee Width from Day 0 (mm)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (1mg/kg/day) + ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (0.3mg/kg/day)
B.
Gait Score
D
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (1mg/kg/day) + ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (0.3mg/kg/day)
C.
Change in Right Knee Width from Day 4 (mm)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (3mg/kg/day; Days 4-28)
D.
Gait Score (change from Day 4)
D
Days
drug-free arthritic
AcF-[OPdChaWR] (3mg/kg/day; Days 4-28)

FIGURE 4.


A.
Intra-articular [macrophage] (10^4 cells/mL lavage)
60
50
40
30
20
10
0
*
saline
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (3mg/kg/day) Days 4-28
AcF-[OPdChaWR] (1mg/kg/day) + ibuprofen (30mg/kg/day)
B.
Histopathological Score
4
3
2
1
0
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
ibuprofen (30mg/kg/day)
AcF-[OPdChaWR] (3mg/kg/day) Days 4-28
AcF-[OPdChaWR] (1mg/kg/day) + ibuprofen (30mg/kg/day)

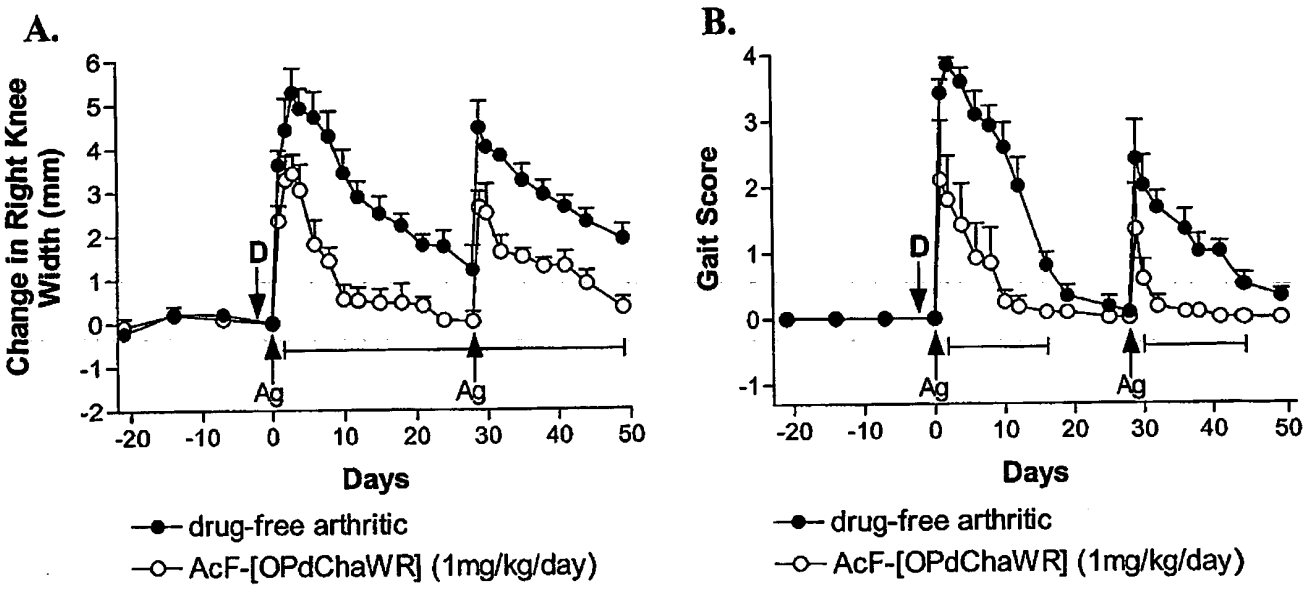
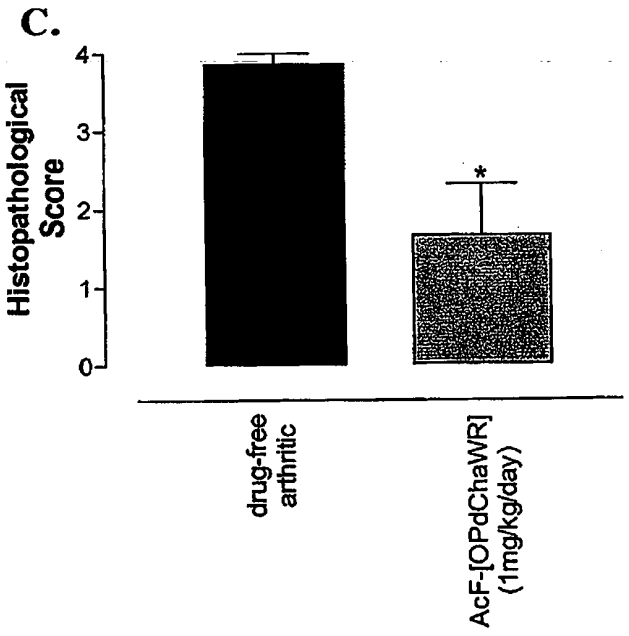

A.
Change in Right Knee Width (mm)
D
Ag
Ag
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)
B.
Gait Score
D
Ag
Ag
Days
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)

FIGURE 5.


C.
Histopathological Score
*
drug-free arthritic
AcF-[OPdChaWR] (1mg/kg/day)

REFEERENCES:

Brackertz D, Mitchell GF and Mackay IR (1977). Antigen-induced arthritis in mice. I. Induction of arthritis in various strains of mice. *Arthritis Rheum.* **20:** 841-50.

Brodeur JP, Ruddy S, Schwartz LB and Moxley G (1991). Synovial fluid levels of complement SC5b-9 and fragment Bb are elevated in patients with rheumatoid arthritis. *Arthritis Rheum.* **34:** 1531-7.

de Ceulaer C, Papazoglou S and Whaley K (1980). Increased biosynthesis of complement components by cultured monocytes, synovial fluid macrophages and skynovial membrane cells from patients with rheumatoid arthritis. *Immunology.* **41:** 37-43.

Dumonde D and Glynn L (1962). The production of arthritis in rabbits by an immunological reaction to fibrin. *Br J Exp Path.* **43:** 373-83.

Hogasen K, Mollnes TE, Harboe M, Gotze O, Hammer HB and Oppermann M (1995). Terminal complement pathway activation and low lysis inhibitors in rheumatoid arthritis synovial fluid. *J Rheumatol.* **22:** 24-8.

Jose PJ, Moss IK, Maini RN and Williams TJ (1990). Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in the acute inflammatory phase. *Ann Rheum Dis.* **49:** 747-52.

Kemp PA, Spragg JH, Brown JC, Morgan BP, Gunn CA and Taylor PW (1992). Immunohistochemical determination of complement activation in joint tissues of patients with rheumatoid arthritis and osteoarthritis using neoantigen-specific monoclonal antibodies. *J Clin Lab Immunol.* **37:** 147-62.

Kiener HP, Baghestanian M, Dominkus M, Walchshofer S, Ghannadan M, Willheim M, Sillaber C, Graninger WB, Smolen JS and Valent P (1998). Expression of the C5a receptor (CD88) on synovial mast cells in patients with rheumatoid arthritis. *Arthritis Rheum.* **41:** 233-45.

Konttinen YT, Ceponis A, Meri S, Vuorikoski A, Kortekangas P, Sorsa T, Sukura A and Santavirta S (1996). Complement in acute and chronic arthritides: assessment of C3c, C9, and protectin (CD59) in synovial membrane. *Ann Rheum Dis.* **55:** 888-94.

Kourounakis L, Nelson RA, Jr. and Kupusta MA (1973). The effect of a cobra venom factor on complement and adjuvant-induced disease in rats. *Arthritis Rheum.* **16:** 71-6.

Makinde VA, Senaldi G, Jawad AS, Berry H and Vergani D (1989). Reflection of disease activity in rheumatoid arthritis by indices of activation of the classical complement pathway. *Ann Rheum Dis.* **48:** 302-6.

Morgan BP, Daniels RH and Williams BD (1988). Measurement of terminal complement complexes in rheumatoid arthritis. *Clin Exp Immunol.* **73:** 473-8.

Neumann E, Barnum SR, Tarner IH, Echols J, Fleck M, Judex M, Kullmann F, Mountz JD, Scholmerich J, Gay S and Muller-Ladner U (2002). Local production of complement proteins in rheumatoid arthritis synovium. *Arthritis Rheum.* **46:** 934-45.

Ruddy S and Colten HR (1974). Rheumatoid arthritis. Biosynthesis of complement proteins by synovial tissues. *N Engl J Med.* **290:** 1284-8.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-7

This is Exhibit SMT-7 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Toni Russ

JUSTICE OF THE PEACE (QUALIFIED)
DEPT. OF JUSTICE & ATTORNEY-GENERAL
No. 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

Guidance for Industry

Clinical Development Programs for Drugs, Devices, and Biological Products f the Treatment of Rheumatoid Arthritis (RA)

[PDF version of this document]

Additional copies of this guidance document are available from:

Office of Training and Communication, Division of Communications Management
Drug Information Branch, HFD-210
Center for Drug Evaluation and Research
Food and Drug Administration
5600 Fishers Lane, Rockville, MD 20857
(Phone 301-827-4573)
Internet: http://www.fda.gov/cder/guidance/index.htm

or

Office of Communication, Training and Manufacturers Assistance, (HFM-40)
Center for Biologics Evaluation and Research (CBER)
Food and Drug Administration
1401 Rockville Pike, Rockville, MD 20852-1448
Internet: http://www.fda.gov/cber/guidelines.htm
CBERFAX or 301-827-3844
(Voice Information) 800-835-4709 or 301-827-1800.

or

The Division of Small Manufacturers Assistance (DSMA), HFZ-220
Center for Devices and Radiological Health
Food and Drug Administration
1350 Piccard Drive, Rockville, MD 20850
1-800-638-2041 or 301-443-6597
Internet: DSMA@CDRH.FDA.GOV
Fax: 1-301-443-8818
Facts-On-Demand (FAX) at 1-800-899-0381 or 301-827-0111

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)
Center for Devices and Radiological Health (CDRH)
February 1999

TABLE OF CONTENTS

Guidance for Industry[1]

Clinical Development Programs for Drugs, Devices, and Biological Products for th Treatment of Rheumatoid Arthritis (RA)

This guidance document represents the Agency's current thinking on Clinical Development Programs for Drugs, Devices, and Biological Products Intended for the Treatment of Rheumatoid Arthritis. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statute, regulations, or both.

I. INTRODUCTION

This guidance is intended to assist developers of drugs, biological products, and medical devices intend for the treatment of rheumatoid arthritis (RA). The document discusses the types of label claims that car considered for such products and provides guidance on the clinical development programs to support th claims.

The central purpose of label claims is to inform prescribers and patients about the **documented** benefit a product. Because RA is a chronic, symptomatic disease that can result in a variety of outcomes with different chronologies, severities, and overall patient effects, any number of different clinical outcomes c provide the basis for a label claim.

Relief of symptoms the C *signs and symptoms claim* C is a central therapeutic effect of most RA therapeutics marketed circa 1997. The claim structure proposed in this document, however, incorporate wider range of patient outcomes than previously allowable RA claims. As a result, guidance is provided demonstrating patient benefit of greater magnitude than is needed for a claim of symptomatic relief. For example, the claims *major clinical response, complete clinical response, and remission* (the same criteri *complete clinical response* while off all antirheumatic drugs) reflect enhanced effects on the signs and symptoms of disease. The claim *prevention of structural damage* is documented by various radiographi techniques. The claim *prevention of disability* is intended to reflect longer term benefits on disease cour The claims and clinical development programs discussed in this draft guidance for industry represent th current views of Agency rheumatologists about achievable and clinically relevant overall outcomes that be evaluated in clinical trials.

Traditionally, RA therapeutics have been categorized as *disease modifying antirheumatic drugs* (DMAR or as *nonsteroidal anti-inflammatory drugs* (NSAIDs). As a result of the ongoing search for more effectiv therapeutics that have a positive impact on the natural history of the disease, promising new therapies a currently being tested in the clinic. Many of the novel agents under study for the treatment of RA defy categorization by putative mechanism of action. As a result, the usefulness of classifying them in the traditional manner may be limited. For this reason, information being provided in labeling about the onse and duration of action and the durability of response of therapeutic interventions is intended to reflect th data that were gathered in clinical trials. Because of this, some of the claims described in this document incorporate response duration times within their structure.

Over the past decade, there has been a search for better measures to describe patient outcomes in RA clinical trials. A number of organizations, including the European League Against Rheumatism, the International League Against Rheumatism, the American College of Rheumatology (ACR), and the Outc Measures in Rheumatoid Arthritis Clinical Trials (OMERACT) group, have attempted to define core grou of measures, as well as composite indices, that describe patient outcomes. As a result of these efforts, several new measures have been described and validated with clinical data. With the hope that these measures will provide more useful information about patient outcomes, this document provides guidanc about the use of these new measures in clinical trials to support label claims.

One outcome measure that is not fully relied upon as a stand-alone claim is general *health-related quali life* (HR-QOL). Since RA affects so many domains of a patient's life, it is hoped that such HR-QOL meas may provide an integrated assessment of the long-term impact of intervention. However, not enough information is available on the performance of general HR-QOL measures in longer term arthritis trials. incorporation of such measures in planned trials is encouraged.

Table of Contents

II. NEW CLAIMS FOR THE TREATMENT OF RA

A number of new claims are now being evaluated in clinical trials during drug development. Descriptions the claims and acceptable outcome measures to support each claim are discussed in the following secti

A. Reduction in the Signs and Symptoms of RA

This claim is intended to reflect the demonstration of symptomatic benefit or benefits that include improvement in signs of disease activity as well as symptoms. Reduction in signs and symptoms ordinarily be the initial claim granted for marketing approval. Ordinarily, this claim is established t trials of at least six months' duration, unless the product belongs to an already well-characterized pharmacologic class (e.g., NSAIDs) for which trials of three months' duration are sufficient to establish efficacy for signs and symptoms. Six-month trials are desirable for several reasons. Fir RA is a disease of long duration. Interventions that provide only short-term, time-limited benefit a unlikely to have overall value to patients. In addition, products with the potential to elicit antibody formation should be assessed for durability, since antibodies may block effectiveness. In evaluati signs and symptoms, methods that evaluate response over time are preferable to methods that incorporate only the baseline value and the final observation, unless there is a reason to weight symptoms at the last visit more than intermediary symptoms. Acceptable outcome measures thal would support claim A include:

1. Validated composite endpoints or indices of signs and symptoms

   These composites may be used to construct categorical endpoints for patient success or failure. For example, the Paulus criteria (1990) or the ACR definition of improvement (ACF [2] could be used to assess a patient's response.

   Illustration: Success for each patient in a six-month trial could be defined as meeting the criteria for improvement over baseline in at least four of six monthly observations and not dropping out because of toxicity

2. Well-accepted sets of signs/symptoms measures

   The four measures previously recommended in the 1988 CDER *Guideline for the Clinical Evaluation of Anti-Inflammatory and Antirheumatic Drugs* (FDA 1988) C joint counts: pain, tenderness, and swelling and global assessments: physician and patient C or the ACR co set are examples of well-accepted sets of signs and symptoms measures. The criteria for success and the methods for statistical analysis should be prospectively defined and agre upon. For example, historically, in using joint counts and global assessments, a statistical significant difference between the control and the treatment group in change from baseline at least three of the four measures has been used as the criterion for a successful trial. However, as stated above, comparison of only the baseline and last observation may not l the best way to construct the analysis since this method leaves out all intervening efficacy observations.

   For both the above measures, using 66 or 28 joint count is appropriate (Smolen 1995).

B. Major Clinical Response

This claim is intended to reflect the demonstration of a continuous six-month period of success b "ACR 70," which is defined entirely parallel to the ACR 20, except 70 percent improvement, rathe than 20 percent, is needed for the component assessed. This claim is based on statistically signi improvement in response rates by the continuous six-month ACR 70 definition compared to background therapy in a randomized control group. For reference, the number of patients satisfyi various definitions of ACR responses from ACR 20 to ACR 70 in two historic databases are giver Appendix A of this document. Trial duration should be a minimum of seven months for an agent expected to have a rapid onset of action and longer for agents with less prompt effects.

C. Complete Clinical Response

This claim is intended to describe a therapeutic benefit of greater magnitude than the *major clinic response* claim. *Complete clinical response* and *remission* (see below) are identically defined as continuous six-month period of both *remission by ACR criteria* and radiographic arrest (no radiographic progression [Larsen 1977] or modified Sharp methods [1985]). Complete clinical response connotes a benefit requiring ongoing drug therapy; *remission* is defined by the same re while off all antirheumatic drugs. The 1981 ACR remission criteria (Pinals 1981) require at least f of the following: morning stiffness less than 15 minutes, no fatigue, no joint pain by history, no joi tenderness or pain on motion, no swelling of joints or tendon sheaths, and erythrocyte sedimenta rate (ESR) less than 20 for males or less than 30 for females. The duration of trials designed to

support this claim will vary depending upon the rate of onset of effect of the test product. For all t the most rapid-onset agents, trials of one year's duration should be planned. Longer trials may b needed for very slow-acting agents. Trials evaluating complete clinical response would use a categorical endpoint (patient complete response or treatment failure) as the primary outcome measure.

D. Remission

This claim is defined as both *remission by ACR criteria* and *radiographic arrest* (no radiographic progression by Larsen or modified Sharp method) over a continuous six-month period while off a antirheumatic therapy. Remission is not intended to imply cure, and a remission claim could be granted even if patients relapse after six months or more of remission. The duration of trials desi to support a remission claim will vary, depending upon the rate of onset of effect of the test prod Ordinarily such trials should be at least one year in duration, and longer trials may be needed for slow-acting agents.

E. Prevention of Disability

This claim is intended to encourage long-term trials in RA. Currently, the Health Assessment Questionnaire (HAQ) (Fries 1982) and the Arthritis Impact Measure Scales (AIMS) (Meenan 198 are adequately validated measures for use as the primary outcome measure in these trials. Studi should be two to five years in duration. Sponsors seeking this claim should plan to have demonstrated previously, or to demonstrate concomitantly, improvement in signs and symptoms. Since the full effect of RA on a patient is not captured without the use of more general HR-QOL measures, a validated measure such as the SF-36 should also be collected and patients should worsen on these measures over the duration of the trial.

F. Prevention of Structural Damage

Prevention of structural damage is an important goal of RA therapy. Trials evaluating this outcom should be at least one year in duration.

The following are examples of outcome measures that could be used to support prevention of structural damage claims.

1. Slowing X-ray progression, using either the Larsen, the modified Sharp, or another validat radiographic index

   Radiographic claims should be based on comparisons of films taken at one year (and subsequent yearly points) with those taken at baseline. All randomized patients should ha films at both time points, regardless of whether they are continuing treatment. Patients dropping out of the trial should have films taken at that time. Prespecification of the handli dropouts is especially important in these trials.

2. Prevention of new X-ray erosions C maintaining an erosion-free state or preventing new erosions

   Trials evaluating this claim would ordinarily use a categorical endpoint to assign a status c progression or nonprogression to each patient, comparing the final state to the baseline st

3. Other measurement tools (e.g., MRI)

   Other measures, such as MRI (magnetic resonance imaging) or ultrasonography, could be employed. However, because of the technique's potential for identifying small, albeit statistically significant changes, the magnitude of the difference that would reflect actual patient benefit is unclear and needs to be established.

Because slowing of radiographic progression does not in itself define a patient benefit, it is expec that the claim of prevention of structural damage would be submitted for an agent that has been shown (previously or concomitantly) to be effective for one of the other claims (e.g., prevention o disability). However, some agents are not intended to affect acute inflammation, but are designe

prevent or slow joint destruction by other means. The first indication that such an agent is clinical useful might be slowing of radiographic progression. Nevertheless, the ultimate goals of slowing j destruction are to improve symptoms and to preserve functional ability. Therefore, slowing radiographic progression of disease is considered a surrogate marker for overall patient benefit ir

Under 21 CFR 314, subpart H and 21 CFR 601 subpart E, FDA can approve drugs intended to tr serious and life-threatening diseases based on an effect on a surrogate marker, provided that cei criteria are met and that there is a commitment to define the actual clinical benefit of the agent in studies completed after marketing. A demonstration of significant slowing of radiographic progres in a seriously affected population of RA patients would qualify for consideration under these regulations. Sponsors are urged to consult with the relevant FDA staff before embarking on a clir program based on these regulations.

One example of a significant effect on radiographic progression might be the demonstration, in a randomized controlled trial, of maintenance of an erosion-free state in a large majority of treated patients when control patients develop multiple erosions. The methods of measurement of the radiographic finding, the magnitude of change considered to be clinically significant (whether a b patient measure or treatment group means), and the methods of statistical analysis should be prospectively defined in the clinical protocol, and the sponsor should seek Agency concurrence v the plan for evaluating efficacy. The use of the *accelerated approval* pathway would necessitate timely completion of phase 4 studies using acceptable clinical endpoints evaluating signs and symptoms or prevention of disability. It is anticipated that these investigations would be extensior the one-year studies used for the *accelerated* approval.

Table of Contents

III. CONSIDERATIONS IN RA PRODUCT DEVELOPMENT

The following information on preclinical and early clinical product development pertains primarily to pharmaceuticals (drugs and biologics). Except in the first two sections, the general principles outlined b also apply to devices. For information specific to the development of devices, refer to the section in this document entitled "Special Considerations for Medical Devices." Developers of products that combine therapeutic modalities (e.g., biologics and devices) may request assistance from FDA in designating a l center for review of the product. Such requests should be submitted to: Office of the Chief Mediator and Ombudsman (HF-7), Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857.

Frequent encountered issues in RA product development include:

1. Selecting appropriate in vitro (animal or human systems) and in vivo animal models for screening potentially active agents.
2. Designing and performing appropriate preclinical safety studies to support the use of a new mole entity in human volunteers or patients.
3. Balancing the potential need for therapeutic intervention early in the disease course with the nee avoid exposing patients with mild disease to agents that have toxicities or little record of safety.
4. Identifying the potential risks associated with combination therapies, particularly those with share target organ toxicity or potential for pharmacokinetic interactions.
5. Designing adequate and practical long-term safety monitoring.
6. Designing trials that definitively show clinical efficacy.

The following sections discuss approaches to the above issues.

A. Preclinical Considerations

This section focuses on preclinical issues that are specific to the clinical development of

antirheumatic therapies. In designing toxicity studies and the timing of such studies, consultation the Agency is recommended concerning the current recommendations and guidances that addre drugs, devices, and biological products. Guidance on preclin1ical safety testing, addressing the r for and design of toxicokinetic, reproductive toxicity, genotoxicity, and carcinogenicity studies has been developed by the International Conference on the Harmonization (ICH) of Technical Requirements for Pharmaceuticals. Because biologics can pose unique challenges in animal stu design (for example, species-specific binding or immunogenicity of human proteins in animals), a specific ICH document is available that addresses the safety evaluation of biotechnology-derived pharmaceuticals (ICH S6 1997).[3]

1. Pharmacokinetics

Animal studies of drug absorption, distribution, metabolism, and excretion are important d the early investigational new drug (IND) phase to aid in toxicity study interpretation, but ne not all be completed prior to phase 1. Generally, for initial studies in humans, determining pharmacokinetic (PK) parameters, such as area under the curve (AUC), maximum concentration (C_{max}), and half-life ($t_{1/2}$) in animals, is sufficient to provide a basis for predi safe clinical exposure.

In the past, preclinical testing of combinations of drugs (or biologics) to be used in patients RA has not often been done prior to the initial clinical trials. However, given the variety of drugs, including NSAIDs, analgesics, corticosteroids, and DMARDs currently used to treat patients, it would be useful to consider this testing of common combinations both preclinic and clinically. In addition, to evaluate potential interactions, information on the impact of concomitant therapies on pharmacokinetics may be needed to optimize dosing regimens a to identify potential safety concerns. Metabolic interactions often may be assessed in an ir vitro system using animal or human liver slices, microsomal preparations, or purified P450 enzymes (FDA 1997).

Interactions may also result from the presence of individual- or disease-specific factors, su as rheumatoid factor, which may bind to various monoclonal antibody therapeutics; in such cases in vitro binding studies that identify patients with high titers may be useful in identify patients who may exhibit unique pharmacokinetics or patterns of clinical response.

2. Biological activity

The biological activity of a potential antirheumatic therapy should be established using mu preclinical model systems (i.e., in vitro, in vivo, ex vivo). In vitro screens can use cells or tissues derived from animal or human sources and are generally used to select candidate drugs that have a desired effect on a molecular target. Such assays can also be used to devise appropriate bioassays for the selected agent. Animals, either healthy, with rheumat disease (spontaneous or induced), or genetically modified, are subsequently used to determine whether the biological effect can be demonstrated in vivo. While the in vivo syst used should mimic one or more aspects of rheumatoid arthritis or its etiology, it is expecte that each animal model will have limitations.

a. In vitro

Data from in vitro studies can be useful in defining the potential mechanism of actio a drug or biologic and for determining relevance of a particular animal species for ir assessment of activity or safety. These data are especially useful if a potential surr marker can be identified in preclinical studies. For example, if the product is intende affect the CD4 receptor on lymphocytes, this receptor can be used as a surrogate marker for both activity and certain toxicities.

Several in vitro tests could be used, depending on the mechanism of action of the c or biologic. For example, binding assays may be useful for developing receptor antagonists or monoclonal antibodies. In vitro functional assays (e.g., platelet and neutrophil aggregation) may be useful tests for identifying inhibitors of inflammatory mediators. Enzymatic assays (e.g., in vitro or ex vivo inhibition of cyclooxygenase, lipoxygenase, and phospholipase) may also be useful for determining selectivity for inhibition of isozymes.

b. In vivo Selection of animal models should be made on the basis of pharmacodynan (PD) responses, similarity of animal disease etiology to clinical disease, and/or to d mechanism-based toxicity. Ideally, products that are targeted for a subset of arthriti patients should be developed in an experimental model(s) that is most relevant to tl subset. For example, rats are not sensitive to drugs that inhibit 5-lipoxygenase. Therefore, mouse or rabbit models are more relevant to evaluate the anti-inflammat activity of leukotriene inhibitors.

The development of rheumatic disease models to allow screening for potential RA candid drugs is encouraged. The following examples are meant only to illustrate some models in current use and are not intended to suggest excluding the use of others.

Collagen-induced arthritis (CIA):

Collagen-inducted arthritis is often considered to be a suitable model for studying potentia drugs or biologics active in human rheumatoid arthritis because of the involvement of loca major histocompatibility, complete class II-restricted T helper cell activation, and the simila of histopathological lesions. Radiographs of joints affected by CIA often show erosive chai similar to those seen in human rheumatoid arthritis. The progressive arthritis often results RA-like joint deformity and dysfunction. Anticollagen antibodies, which occur in some patie with RA, develop in the CIA model.

The CIA model has been useful for assessing immunosuppressants and steroid hormones well as inhibitors of inflammatory mediators. Since this model can be induced in several ar species, it may be especially useful for evaluating drugs that are species-specific (e.g., leukotriene antagonists and 5-lipoxygenase inhibitors). In addition, although functional tes are not routinely used in this model, incorporation of measures of mobility and joint functio may enhance the predictive value of the model.

Naturally occurring arthritis or autoimmune response:

MRL/lpr mice, Biozzi H mice and DBA/1 mice have been used to examine the onset of dru induced tolerance and immunosuppressant drug effects on autoimmunity. The MRL/lpr mc model has been useful for evaluating immunosuppressants and hormones.

Rat carrageenin-induced acute model of inflammation:

This model has been useful in assessing anti-inflammatory activity of cyclooxygenase inhibitors. Most of the animal models that involve inflammation in the paw may be used for measuring antiphlogistic action of a drug.

Adjuvant-induced arthritis in rats (AA):

AA in rats has been frequently used for screening nonsteroidal anti-inflammatory drugs an inhibitors of inflammatory cytokines as well as antimetabolite-like immunosuppressants.

Streptococcal cell wall-induced arthritis:

This model has been used for developing cytokine inhibitors.

Experimental organ transplant in animals:

This model has been used to identify the activity of immunosuppressants and antimetaboli particularly those directed at cytolytic cellular immune processes.

Transgenic animal models:

A number of transgenic animal models are being developed for the study of rheumatoid arthritis and may prove useful over the next decade. Some examples include transgenic m that carry genes for the env-Px region of the human T cell leukemia virus type I genome, human TNF, CD4, and HLA B-27.

3. Toxicology

Preclinical toxicology studies of a drug or biological product are designed to characterize general and specific toxicity using dosing routes and regimens as similar as possible to th proposed clinical trials with consideration of the demographics and disease status of the intended patient population. For instance, the prevalence of RA is high in females. Therefc reproductive toxicity studies should be completed early in clinical development to support inclusion of women of childbearing age in early phases of clinical trials. The need for reproductive studies for biological products is likely to be case-specific due to complicatior arising from immunogenicity and species selectivity. Therefore, standardized study design such as those recommended in the ICH reproductive toxicology guidance, may not be fea or clinically relevant for biologics (ICH S5 1994). The need, and specific designs, for these studies may be discussed with Agency review staff.

Immunomodulatory or immunosuppressive agents administered to RA patients as monotherapy or in combination raise concerns about the adverse effects of prolonged immunosuppression. For example, malignancies (i.e., lymphomas) are a known risk of lon term, nonselective immunosuppression used for treatment of graft recipients. Investigatior drug-related opportunistic infections and mortality related to immunosuppression have occurred in RA patients. Sponsors are encouraged to identify and use animal models that assist in selecting drug candidates that selectively inhibit cells and processes responsible RA.

Antirheumatic drugs are often used in combination in an attempt to improve outcomes anc minimize toxicities. However, drug interactions may result in increased toxicity, even at lov than previously evaluated doses of either agent. This concern is especially evident for age that have long half-lives or nonselective activity, or for drugs that share common target orc toxicity. Preclinical toxicity studies that evaluate the use of combined agents may be helpfi predicting clinical safety hazards. The duration of toxicity dosing of animals is usually linke patient dosing regimens. Development and validation of in vitro or whole animal models is encouraged to address concerns regarding short- or long-term toxicity and to identify surrc markers for patient immunocompetence.

B. Pharmacokinetic/Pharmacodynamic Strategies

In vivo pharmacokinetic studies should be used to evaluate drug disposition and metabolism, dec of linearity and accumulation, dose proportionality, and, for oral dosage forms, food interactions (1992). Some of these data can be gathered in a single study designed to evaluate a number of parameters. During formulation development, bioequivalence studies linking formulations may be recommended.

A particular concern with biological agents is the development of antibodies that may accelerate clearance or alter its distribution, resulting in changes in therapeutic benefit over time, or followin repeated courses of treatment. To address this consideration, it is desirable for sponsors to build their repeat-dose clinical protocols a coordinated evaluation of drug levels, receptor saturation, antidrug antibodies, and clinical responses. Optimally, these assessments would be conducted a initiation of therapy and at several time points over the course of therapy. The presence of antidr antibodies and their role in altering drug exposure, clinical activity endpoints, or adverse events v be evaluated. The goal of an integrated analysis of these parameters is to provide data to guide dosage or schedule changes to optimize therapeutic benefit. The best time for conducting these pharmacokinetic studies is prior to phase 3, before commitments have been made regarding dos and schedule.

Because polypharmacy is common during the treatment of rheumatic disorders, in vitro binding studies with blood from patients with active disease should be used as a preliminary screening tc for potential displacement reactions.

For products that may interact with rheumatoid factors (e.g., monoclonal antibodies), the frequen patients with rheumatoid factor reactive to the antibody, as well as the effect of interactions on th pharmacokinetics of the product, should be evaluated.

C. Considerations in Phase 1 Trials

For general information on clinical development pertaining to most drugs and biological products, the CDER guidance *General Considerations for the Clinical Evaluation of Drugs* (FDA 1978).

The term *phase 1* has two connotations: one refers to the earliest, first-time-into-humans trials, w the other encompasses studies of pharmacokinetics, metabolism, drug interactions, special populations, and the other clinical pharmacology trials as described above. It is expected that bot kinds of phase 1 trials ordinarily will be conducted during the clinical evaluation of therapies for R This section is primarily intended to discuss issues related to the first time people are exposed to drug (including to a particular dose level, combination, or duration of therapy).

1. Settings and investigators

   First-time-into-humans phase 1 studies should be carried out in institutions with a full rang clinical and laboratory facilities, and the patients should be kept under close observation. I desirable that the trials be under the direction of physicians with experience in early drug development and rheumatology, or that a team of investigators combining experience in rheumatology and clinical pharmacology be employed.

2. Subjects First-time-into-humans drug trials are frequently conducted in healthy volunteers. Such studies are predicated upon the ability to perform and to interpret the results of precl animal tests. If the preclinical testing does not reveal potential mutagenic or immune syste effects, or potentially serious effects at or near the expected therapeutic range, testing in volunteers may be initiated. However, for products that have potentially serious toxicities, i may be appropriate for initial testing to be performed in patients with some potential to ber This has created challenges in selecting an appropriate initial patient population.

   For products that have been tested in relevant preclinical toxicity evaluations and have be found relatively safe (without the potential for mutagenic, immune system or other serious effects at the proposed doses), trials may be initiated in healthy volunteers. However, if significant effects have been demonstrated or might be possible, selection of an appropria patient population is necessary. It is recommended that patients meet the ACR criteria for diagnosis and activity of RA and be without other serious medical conditions. Patients with minimal disease are sometimes not appropriate for the same reasons that the testing is nc initiated in healthy volunteers. Patients with devastating RA may also not be the best starti population because of the medical complications of their disease. In addition, they may be likely to respond to therapy.

   There is ongoing epidemiologic work on identifying markers of increased risk in RA. These could be useful for identifying patients with poor prognoses, who might be considered for v aggressive treatments of potential high toxicity (e.g., immunoablative therapies followed b stem cell transplants). Application of epidemiologic studies may allow a very aggressive treatment to be restricted to a subset of RA patients who have a demonstrated shortened span due to their disease (e.g., subjects with greater than 30 affected joints or a HAQ scor with fewer than 75 percent of questions answered "without difficulty").

   When the characteristics of the agent suggest that it may potentially have long-term gonac effects, it is desirable that men and women not wishing to parent children be chosen for pl 1 studies.

3. Trial design

   Ordinarily, initial phase 1 studies are sequential dose escalation trials, in which safety and tolerance at a specific dose is established before exposing additional subjects to a higher dose. A single dose is almost always tested first, followed by repeated dose studies; howe this design is influenced by the type of agent used. Although escalating the dosage to a cl determined maximum-tolerated-dose (MTD) will aid future trial design, in some instances i not medically prudent to try to fully characterize the MTD. Additionally, for some products, MTD may be undefinable.

   The starting dose chosen is often a no-adverse-effect dose (determined by interspecies milligrams (mg)/meter square/day dose conversion from animal to human). For biologics, t initial dose chosen is often one thought to have no adverse biological effect, with caution

regarding the possibility of relative species specificity and comparing receptor avidity betw test species and humans. Conservative dose escalations (e.g., half log or less) are usuall recommended.

4. Concomitant therapy

Use of low-dose corticosteroids (up to 10 mg prednisone equivalent daily) and NSAIDS m ordinarily be continued in phase 1 trials. Concomitant therapy with methotrexate and simil agents should be avoided in initial phase 1 trials of all novel antirheumatic drugs, biologics and devices because of the difficulty of differentiating the toxicity of the novel agent from t of the co-administered product.

Physicians now prescribe methotrexate and similar agents earlier in the course of rheuma arthritis. Recruiting adequate numbers of patients not taking these agents may be difficult. Approaches that may allow the use of methotrexate and similar agents in later phase 1 tri include (a) obtaining reassuring evidence of lack of toxicity from relevant animal models in which co-administration occurred and (b) starting at doses significantly lower than the no-adverse-effect level of the single agent as determined by earlier phase 1 studies or preclin studies, or both. Such proposals should be discussed in the planning stages with Agency

5. Observations

a. Safety

The standard batteries of safety observations have been described elsewhere (ICH 1994). However, additional types of safety observations may be necessary (e.g., te of effects on cellular and humoral immune function or host defenses). For products the potential for effects lasting long after administration, or for delayed toxicity, appropriate follow-up should be designed. For example, phase 1 studies of agents to deplete or modify the function of T-cell subsets should be designed to carefully assess both the short- and long-term effects on number and functional status (e.g., delayed type hypersensitivity responses) of cell populations and other pertinent pharmacodynamic assays during therapy and during follow-up.

It is also desirable to incorporate individual patient adverse event stopping/withdrav rules into protocol designs. In addition, it is often advisable to incorporate into trial designs rules for trial stopping or trial modification in case adverse events are obse For example, dose escalation rules should be clearly defined in dose-finding studie with provisions for enrollment of additional patients at or below the dose-causing to if possible significant adverse events are observed.

It is desirable to develop a standardized toxicity grading scale for use in all trials of product based on the known and suspected toxicities of the product or of the drug This may improve consistency of adverse event reporting and allow more accurate comparisons among trials.

b. Efficacy

Developing an understanding of the agent's therapeutic potential in early trials is hi desirable for efficient product development. This may be attempted in phase 1, but ordinarily be achieved only by performing controlled trials. RA responses in open tri are of little value in indicating efficacy. Consideration should be given to the more modest goal of determining whether the pharmacological effect predicted from the preclinical development is present (proof of concept).

D. Considerations in Phase 2 Trials

During phase 2, larger, often longer, term trials are employed to better define the dose- and exposure-related activity and toxicity of the agent. Enough information should be generated to en that the phase 3 trials can be conducted safely and with a probability of success. In addition, pha trials should solidify a total drug development strategy to ensure that, after the phase 3 safety/effi trials are done, all of the information needed for registration will have been gathered, including ar

appropriate safety database, clinical pharmacology, dose-response data, the exploration in speci populations (e.g., renal failure, hepatic failure, pediatric patients), and information on drug interac with agents expected to be co-administered.

There is nothing to preclude conducting additional phase 1 clinical pharmacology studies and ph trials while the phase 3 development is ongoing.

The following issues are important for phase 2 trials in RA:

1. Trial design

   Dose finding is a central challenge of phase 2 development. Once a reasonably safe rang doses has been established, randomized, parallel-arm dose-comparison trials are ordinari recommended. The use of a placebo arm is desirable for several reasons. First, if no difference is found among doses, there is usually no other way to determine whether all d were equally effective or equally ineffective. Second, if a dose-response trend is found, th placebo arm may indicate the possible magnitude of the observed effect. If use of a placet impossible, designs should include wide dose ranges or durations, or repetitions. Active controlled designs that specify an arm with a well-characterized, known therapy can also b very useful.

   Signs and symptoms measures may be used for dose-finding studies, but it is possible tha separate dose-finding studies may be needed for longer term endpoints.

   For agents that are thought to have prompt onset and rapid offset of effect, alternative des including crossover and titration designs, may be useful, although historically this has not l the case. Trials of two or more doses that permit liberal titrating per the patients' response unlikely to clearly demonstrate a dose response because these titration designs result in a blurring of any existing dose distinction.

   The desirability of identifying a range of doses with acceptable toxicity and reasonable act for study in phase 3 cannot be stressed enough.

2. Safety

   Every RA investigational therapy raises safety concerns. Whenever there is a potential for significant toxicities, long-lasting or delayed-onset, it is desirable to design the phase 2 stu to provide a group of patients with longer term follow-up preceding the larger phase 3 stud Provisions for long-term follow-up can be helpful in addressing issues raised during prema review (e.g., potential for immunosuppression, opportunistic infections, neoplasia, and induction of autoimmune disease). Standard toxicity grading scales and stopping rules are desirable in phase 2.

3. Additional development aspects

   a. Concomitant therapy

      Before starting phase 3 trials, an evaluation of the test product's interaction with oth agents likely to be used by the target population should be performed. Initial inform can be established based on metabolic pathways, studies of in vitro systems, anim human pharmacology studies, or drug interaction studies. This type of information h in directing areas in need of clinical evaluation. When products are intended to be tested as combination therapy with the investigational agent, substantial informatio interactions and safety of co-administration should be developed in phase 2.

   b. Gender effects

      Most RA trials have predominantly female enrollment. Sponsors should evaluate whether the observed safety and efficacy findings are restricted to women or can b also extrapolated to male subjects. This may be accomplished by subset analyses f trials, PK data, or other information (FDA 1993).

E. Efficacy Trial Considerations

The overall goal of phase 3 work is to demonstrate the efficacy of the product in convincing contr trials and to accrue a sufficient safety database. Efficacy trial protocols should contain an analytic plan that precisely identifies the primary comparison(s) to be made, the criteria for success of the and the statistical tests that will be used. Protocols should be designed to generate data that sup desired labeling claims. Any additional planned, ongoing, or completed trials that are also intende generate data supporting the claim should be identified.

1. Global considerations

   a. Patient selection

   Activity of disease: Unless some other specific subgroup is targeted, patients enroll efficacy trials should at a minimum meet the disease definition and disease activity defined by ACR criteria. Consultation with the Agency on the generalizability of clai derived from trials with significant limitations on entry criteria is recommended.

   To enhance the power of the trial, strategies to improve the chances of a response therapy are often employed. Some designs incorporate an attempt to select active patients by withdrawing background treatment and allowing patients to *flare*. Only individuals with sufficiently high scores are enrolled. The relevance of this type of observed flare is questionable, and its ability to predict the normal course of active disease has not been established. Many patients randomized to placebo in such st exhibit the characteristic response of rapidly returning almost to baseline without fu treatment. In addition, when patients undergo blinded withdrawal from therapy withi these trials, similar dramatic flares are not observed. This raises the question of wh there is an expectation bias on the part of patients, who have been told about the fl procedure, and ascertainment bias on the part of investigators, who wish to have patients meet the entry criteria and enroll in the study. These uncertainties and instabilities around the outcome measures used in such trials should be kept in min when employing these designs.

   A proportionately smaller, but nevertheless noticeable and prompt, *regression to th mean* is noted in the joint scores of patients required to have a certain minimum val for trial entry in trials not employing a *flare strategy*. This means that patients, on th whole, will not actually have disease as active as anticipated when the entry criteria set.

   Subgrouping patients by disease markers: RA is likely composed of a number of m or less distinct diseases delineated by a common genetic background, correspondi clinical manifestations, similar serologies, and responses to therapy and prognoses The study of RA possibly may be made more efficient with the use of markers with prognostic significance as entry criteria to increase patient homogeneity. Novel epidemiologic and molecular genetic approaches may lead to identification of even more subgroups. However, prospective studies are first needed to confirm the clinic usefulness of new purported prognostic factors. Where existing data do support markers as prognostic indicators (risk factors), such as the presence of rheumatoid factor, erosive or vasculitic disease, and DR4 homozygosity, they can be taken into consideration in the design of trials, as can factors known to affect treatment respor Although in some cases such studies could limit generalizability and impact labeling the final product, it is also possible that such targeting could improve the risk/benefi profile.

   b. Concomitant antirheumatic therapy

   Studies in RA patients, except in those with very mild disease, are carried out in the presence of concurrent active therapies, including steroids, NSAIDS, hydroxychloroquine, etc. This concurrent therapy creates numerous challenges in patient selection, toxicity monitoring, and clinical trial design. For example, since methotrexate therapy is used to treat many RA patients, it is inevitable that new age will be used in combination with methotrexate in clinical practice unless a contraindication exists. Therefore, unless a prohibition on concurrent methotrexate

supportable, data regarding use of the investigational agent in combination with methotrexate are needed to evaluate the potential for immunosuppression from combination therapy. Other agents should be similarly evaluated.

In addition, patients can be categorized according to their prior responses to standa therapy. Varying trial designs may help assess the response of different response categories to an investigational therapy. For example, with respect to methotrexate the RA population can be divided into five groups: (1) methotrexate noncandidates disease too mild or too early for methotrexate; (2) methotrexate candidates C disea sufficiently (or will become sufficiently) active to justify methotrexate; (3) methotrexa successes C disease reduced to negligible amounts; (4) methotrexate failures C cle drug failures, for inefficacy or tolerability, and (5) methotrexate *partial responders* C considerable residual disease despite methotrexate. Each of these groups might be considered separately for candidacy for an investigational agent and with respect to appropriate trial design. If only a subpopulation of RA patients (e.g. methotrexate nonresponders) is studied in a particular trial, the results strictly reflect efficacy in th group only, but they may, of course, imply something about efficacy in other groups Single trials in various responder subpopulations could be supported by positive res in other subpopulations. Any planned subpopulations should be clinically distinguishable. Sponsors should consult Agency personnel when planning a clinica development program contemplating an RA claim that is limited to a subpopulation the disease.

c. Other concomitant therapies

Most patients with RA are taking concomitant medications. Use of medicines unlike influence treatment outcomes (e.g., antihypertensives) should simply be recorded, although investigators should be alert for possible drug interactions. Obtaining information during clinical development on co-administration of the test medication expected concomitant medications is desirable. The following approaches may be considered in dealing with arthritis medications or analgesics.

Prohibit use: This strategy may result in noncompliance or an increased number of dropouts.

Incorporate protocol-specified use, with monitoring: With this strategy, additional analgesic use (and possibly other arthritis medications) may be used according to protocol-specified criteria. In addition, for long duration studies, protocols should address (1) whether intra-articular steroids are permitted and, if so, for how long assessments of the involved joint are excluded from analysis; (2) the manner in whi stress doses of corticosteroids for surgery, etc., are to be handled; and (3) how soo after such doses protocol assessments would be allowed.

Design analgesic use, or its quantitative consumption, as (part of) an efficacy endp

Define use of more arthritis treatments as (part of) an efficacy endpoint.

d. Stratification

Randomization is intended to balance populations for confounding variables; howev there is always a chance that randomization may fail to achieve balance, particularl smaller trials. It may be advisable to stratify known (or highly suspected) major risk factors to ensure their balance across arms. Any factor whose influence on the outc is suspected to be as strong as the treatment's influence should be considered for stratification (e.g., erosive disease, presence of rheumatoid factor). An often overlo risk factor is the patient's past therapeutic history. (See statistical section for further discussion.)

e. Blinding

Because most RA outcome measures have a high degree of subjectivity, the highe confidentiality in patient and assessor blinding should be sought to achieve a credit

inference. Blinding may be compromised if there is not an approximate parallelism i time to onset, nature of response, and toxicity profile between active and controlled interactions. Trials should have parallel (e.g., "double dummy") dosing in all arms w possible so that a drug requiring frequent dose manipulations does not threaten the blind. If *arm specific* treatment adjustments are necessary (e.g., per monitored drug levels), these can be done by an unblinded (and sequestered) third party to maintai patient and assessor blinding. In this case, parallel changes should be made as dui adjustments in the control arm to preserve blinding. Similarly, if the blind is likely to compromised by infusion-related events or other features of the treatment protocol, critical treatment endpoints, such as joint counts, should be assessed by an independent party with no knowledge of the subject's history.

f. Effects of dropouts and noncompliance.

It is important that trials be designed to minimize dropouts and the attendant inform loss. Traditionally, recommended RA trial designs have focused on eliminating sou of variability, for example, extra pain medications, and intra-articular injections. Ofte these interventions were defined as major protocol violations, requiring that the pati be dropped from the study. There is a trade-off between patient retention and tolera of variability in RA trial design. Protocols demanding rigid adherence may yield uninterpretable results because of dropouts and noncompliance emanating from pa and investigator intolerance of the requirements. On the other hand, protocols permitting any kind of additional intervention may likewise be so confounded as to c interpretation.

The following strategies may help minimize loss of information:

i. Use screening or run-in periods so that patients are randomized to treatment groups only after their eligibility and commitment are confirmed.

ii. Thoroughly train investigators and study personnel to minimize inappropriate enrollments, protocol violations, and other deviations that would decrease th ability to assess trial outcomes.

iii. Include dropouts in the definition of the endpoint, as in a time to defined treatment failure, or a defined by-patient success or failure. A sufficiently late time point should be chosen as the endpoint to avoid the situation where the course for response differs between two active therapies in a trial, which can introduce bias.

One example of this approach would be to use a protocol-defined response as the primary endpoint, wherein dropouts due to lack of efficacy are classifi as nonresponders. With this type of endpoint, the criteria for classification as nonresponder should be clearly and prospectively defined. The use of this ty endpoint could be justified in situations where there are robust phase 2 data suggesting drug responsiveness at a defined point after initiation of therapy a durability of that response. In this case, one could define the primary analysi a comparison of the proportion of patients with an ACR 20 response at six months. The protocol could specify that if no improvement compared to base were seen on two consecutive study visits after two months on protocol, the subject would be declared a nonresponder. Experience will determine wheth this approach effectively limits information loss due to dropouts.

iv. Make provisions for following patients who have stopped experimental treatr Options include allowing a protocol-specified crossover to a standard therap patients meeting predefined criteria for treatment failure.

v. Allow more flexibility in treatment options during the study. Some designs tha have been used include allowing dose adjustment of the comparator arm (assessor and patient blinded), allowing add-on therapy for patients meeting predefined criteria for inadequate response, and allowing a limited number o joint injections, with elimination of that joint from assessment.

2. Trial designs in RA

Clinical trials in RA can be designed in a variety of ways. More than one claim can be purs in the same trial, and claims can be submitted singly or together. Trials can be designed tc a difference C demonstrating that the investigational product is superior to control (placebo lower test dose, another active agent), or they can be designed to test no difference C demonstrating that the product is adequately similar in efficacy to active control. Placebo, dose, concentration- or active-controlled designs can be used.

Because the persuasiveness of trials showing a difference is, in general, much greater tha that of equivalence trials, it is highly desirable for a claim to be convincingly demonstrated least one trial showing superiority of the test agent over placebo or active control. If a clair superiority over a specific comparator is sought, rather than just straightforward efficacy, tl claim should be substantiated by two adequate and well-controlled trials showing superior Such trials can also be the basis for demonstrating the product's efficacy.

a. Superiority trials

The standard two-arm, investigational agent versus placebo design has been the m common RA design and is the most straightforward. The details of trial design will depend on the population tested. Patients with mildly active RA taking only NSAIDS who have never been treated with an additional class of therapy, may be enrolled ir placebo-controlled trial with continuation of NSAID background therapy; however, patients doing poorly on NSAIDs alone are usually not appropriate candidates for placebo-controlled trials. The same considerations apply to patients who are partial responders to, or who have failed, various other treatments.

Alternatives to the two-arm difference design are a standard dose-response study a superior-to-active-control hypothesis. These designs may accommodate the need t provide active treatment to patient groups where randomization to placebo is infeas

b. Equivalence trials

Equivalence trials are designed to demonstrate that the test drug is adequately sim to an active control. This is done using a prospectively defined equivalence test, specifying to a 95 percent confidence level that the real difference between test anc control is smaller than some predetermined amount. Achieving similar point estimat efficacy of two agents is not a demonstration of equivalence.

Equivalence trials can serve two purposes. First, they can be used to supply evider for a simple efficacy claim. Second, they can be used to pursue a specific equivaler to drug X claim. Both purposes could also be pursued in the same trial. The importa point to note is that ***the strength of the evidence may need to be stronger for a claim of equivalence to drug X than for a simple efficacy claim.*** Thus, the equivalence test may differ, depending on which claim is intended. Currently, the equivalence standard that is appropriate for a given trial in RA will be determined o case-by-case basis. As noted above, this test may be more stringent if a claim of *equivalence to drug X* is being pursued. Additionally, the test of equivalence may b constructed differently if a placebo arm is present, since the presence of the placeb allows estimates of absolute and relative drug effect size.

In either case, the statistical test for equivalency needs to be quantitatively describe the protocol. Under either the pursuit of a simple efficacy claim or the pursuit of a specific *equivalence to drug X* claim, the basis of the decision on an appropriate tes remains, fundamentally, a clinical one. It represents a consensus, in that particular circumstance and for that particular claim, on what small potential difference can be considered clinically insignificant, to allow the treatments to be considered clinically equivalent.

There is considerable experience in the interpretation of active-controlled trials in cl situations where the response to the intervention is high. As an example, antibiotics evaluated by the Division of Anti-Infective Drug Products (CDER, FDA). For these

products, the magnitude of the potential difference permitted in an equivalence determination depends on the response rate of the standard treatment. For exampl new agent being compared to standards with response rates of 90 percent or more be allowed a 10 percent window to provide confidence that the difference between standard response rate and the test response rate is no more than 10 percent. Technically, this means the 95 percent confidence interval of the difference must lie to the right of 10 percent. If the standard treatment is known to have an 80 to 90 pe response rate, a 15 percent window is used. These equivalence tests were designe trials without a placebo arm and for clinical situations where the placebo response i known to be very low.

Treatment response rates in RA are often in the vicinity of only 50 percent (dependi of course, on the endpoint used) with placebo rates of about 20 percent, so the clin decision for an allowable small difference may differ from that with antibiotic therap this time, the decision will have to be individualized for each trial.

A major problem in equivalency trials lacking a placebo arm is ensuring that both treatments are equally effective, rather than equally ineffective. A number of the ag that are approved for RA have fairly small effects and may fail to show efficacy whe tested against a placebo. Comparative trials intended to show *equivalence* to such treatments, when not anchored by a placebo control group, may lack credibility. Thi is desirable in equivalence designs to select highly effective comparative agents. If possible, use of a third (placebo or lower dose) arm, so that a treatment difference be shown, is a desirable strategy in equivalence trials. This arm would not necessa have as many patients or as long a duration as the active comparators. If a placebc is present, both the test and active arms need to statistically exceed placebo for a finding of *equivalence* to have meaning.

Strict attention to numerous aspects of trial design and conduct are important to en accurate inferences from equivalency trials. Design decisions regarding patient population, dosing, and efficacy and safety assessments should be done in a mann that is unbiased against the control to ensure a *fair comparison*. Furthermore, atten to certain problems in trial conduct, such as minimizing dropouts, noncompliance, a missing data is essential to the reliability of the inference. These aspects of trial cor may obscure differences and lead to a false conclusion of equivalence. This is the opposite of their effect in a difference design to show superiority, where they work against trial success.

In any particular development, the choice of trial design depends on many factors. Since controlled evidence showing a difference is more persuasive than that showi equivalence, greater efficiency (fewer patients or shorter exposures) is available wi development strategies using trials employing maximal differences between trial an Optimally, this means placebo controls, with the requisite *background therapy,* give all patients.

c. Trial designs novel to the study of RA

Although not used traditionally in the study of new RA treatments, the withdrawal de can be considered in certain circumstances. The withdrawal design is sometimes u to assess efficacy. In this design, patients in both arms of a study are treated with tl investigational agent, which is then blindly withdrawn from one arm, after which pat outcomes are compared. Showing that patients taken off the investigational drug ge worse demonstrates effectiveness. Natural endpoints for withdrawal designs are *tin (predefined) worsening* using standard *time-to-occurrence* statistical tests or a simp comparison of proportion of outcomes in the two arms. Withdrawal studies may be performed with both arms on background therapy.

There are a number of caveats about withdrawal designs. If the product is very toxi that only a small (tolerant) subset of the original population remains at the end of th trial and is available for the double-blind withdrawal phase, the generalization of an inference from the withdrawal design is limited to that tolerant subset. Additionally, i should be noted that, if a drug induces habituation or tolerance, withdrawal or rebo phenomena may make withdrawn patients worse even though drug therapy did not

have a beneficial effect.

3. Analytical Issues

   a. Handling dropouts

      Historically, RA trials have suffered from information loss due to dropouts. Dropouts probably never occur randomly, and rarely occur fully independent of the treatment being tested, so there is always the possibility that dropouts introduce a bias. This problem is common in many randomized trials. Methods for analyzing the effects of dropouts have been proposed, but none is fully adequate.

      The problem of dropouts is not resolved by using an intent-to-treat (i.e., all randomi patients included) analysis with an imputation by last-observation-carried-forward (ITT/LOCF) or by showing that both the ITT/LOCF and PP/OC (per protocol completers/observed cases only) analyses concur, although these approaches may increase confidence in the results. It should also be noted that there are other meth of modeling missing data, for example, see Little and Rubin (1987). Such modeling methods require assumptions that are nonverifiable by existing data.

      The effects of dropouts should be addressed in all trial analyses to demonstrate tha conclusion is robust. One trial design approach is following all patients, including dropouts, to the planned trial endpoint, even if postdropout information is confounde new therapy, and performing an analysis including these patients. Another approac involves the *worst case rule:* assigning the best possible score to all postdropout placebo patients and the worst score to all postdropout treatment patients, then performing an analysis including these scores.

   b. Comparison to baseline outcome measures

      A phenomenon frequently observed in RA, as well as in other conditions, is that pat who stay in trials do better than those who drop out: responders do better than nonresponders. This is true both for placebo groups and active treatment groups. If observations of the disease were made exclusively from clinical trials, one might conclude that the natural history of the disease is inexorable improvement. This phenomenon is attributable to preferential dropout of worsening patients (a phenomenon not adequately compensated for in LOCF analysis) as well as *regress to the mean.* The problem is exacerbated in flare designs, where all patients have r improvement regardless of treatment status. This fact makes comparison-to-baselir outcome measures difficult to assess, since, very often, much of the improvement r has no relationship to a treatment effect. For these reasons, active-controlled trials incorporating a placebo arm and using comparisons to baseline may be extremely difficult to interpret, especially if a flare design is employed. In any case, success in trial implies improvement over control.

4. Statistical Considerations in Efficacy Trial Design

   It is advisable to discuss the design and analysis with the FDA review team prior to embar on a study. In addition, FDA's Guideline for Format and Content of the Clinical and Statisti Sections of New Drug Applications (1988) contains useful information.

   a. Randomization/stratification

      Randomization is intended to allocate patients to treatment groups to avoid bias an ensure that statistical procedures can be appropriately applied.

      In some clinical trials, there are known factors that are at least as influential in controlling the observed severity of disease as the drugs being studied. Stratificatio may be used to avoid relying on randomization properties to balance patient assign for these factors. Stratification is implemented by constraining simple randomizatior balance the assignment of patients to treatment groups for the chosen stratification factors.

Every phase 2 and phase 3 study protocol should contain a randomization section. constraints imposed on the randomization should be explicitly identified. It can then inferred, when a stratification factor or sample size allocation constraint is not mentioned in a protocol, that there exists no corresponding randomization constrair This applies to whether patients are blocked to balance treatment assignment for ti of patient entry into study and to the more obvious stratifications on center and bas

Because stratification implies constraints on randomization, studies that have been stratified for certain factor(s) should account for these factors in the statistical analy section. The protocol-defined analysis should be implemented for each study.

There are also statistical procedures to address bias in treatment group comparisor adjusting for factors (covariates) that, like the stratification factors, are to be prespe in the protocol or by using a mechanism to determine a fixed number of covariates prespecified. It is important to prospectively identify covariates (or criteria for choos covariates) in the protocol. In addition, statistical adjustment procedures should be with care and include appropriate verifications of assumptions.

In deciding whether to stratify randomization in all clinical trials, practical judgment i required. There are reasons to choose stratification and reasons to choose statistic adjustments.

The first advantage of stratification is that it avoids possibly major statistical adjustn of differential treatment effects. Stratification would essentially eliminate the effect c such adjustments before analysis began. Second, although stratification and statist adjustment procedures are both designed to remove bias in estimated treatment ef stratification is usually more powerful. This is because stratification leads to smaller variances of estimated treatment effects than does statistical adjustment without stratification. Finally, the inclusion of stratification factors into a statistical analysis n should result in increased power to detect effectiveness.

Stratification becomes increasingly clumsy as the number of strata increases and, consequently, the available number of randomizable patients per cell decreases. In case, it is logistically simpler to not stratify, but to rely on statistical methods to adju these factors.

The best approach may be to combine stratification, applied to a limited number of most influential prognostic factors, with statistical modeling. This protocol-defined statistical modeling would both account for stratification and be used to adjust for th effects of a parsimonious number of the most important remaining factors

b. Identification of primary efficacy variables

Each phase 2 or phase 3 study protocol should identify the primary and secondary efficacy variables. Primary efficacy variables are critical to the identification of the effectiveness of the product. Secondary efficacy variables are those that support th validity of the primary variables but are less critical in deciding if this product is effe It is helpful, but not necessary, that statistical evidence of efficacy be shown for secondary efficacy variables.

c. Prespecification of statistical analysis

Statistical analysis of clinical endpoints is part of the process for obtaining consiste and convincing evidence of product efficacy. These statistical analyses should not l data driven. This is implemented by identifying, in each study protocol, before data available for analysis, a sufficient description of the statistical analyses of primary efficacy variables so that an independent statistician could perform the protocol analyses. A brief description of the statistical analyses should include but not necessarily be limited to specifying: (1) the level of significance to be used; (2) whe statistical tests of hypothesis or confidence intervals will be 1 or 2 sided; (3) whethe interim analyses are planned and, if so, how the tests of hypotheses and confidenc intervals will be adjusted to account for interim looks at the data; (4) the mathematic

expression of the statistical model(s) used; (5) the minimal statistical results neede demonstrate a successful outcome; (6) the treatment of missing values and dropou (7) the method used for controlling type I error rates for multiple primary efficacy variables; and (8) the method used for making multiple treatment comparisons.

d. Multiple endpoints

There has often been a clinical argument for using multiple endpoints to assess pri evidence of effectiveness in RA. The theoretical bases for such combination endpo are the focus of an area of ongoing statistical research. For example, for the four measures recommended in FDA's previous guidance (FDA 1988), trial results were considered to support a conclusion of effectiveness when statistical evidence of effi was shown for at least three of the four measures: physician global assessment, pa global assessment, swollen joint count, and painful joint count.

Multivariate statistical methods are also available for analyzing the set of primary efficacy variables. Procedures are being developed for inferences derived from mul endpoint results.

Efficacy variables can be combined within patients (composite endpoint). Such a fi combination of efficacy measures should be well defined in the study protocol. Composite efficacy variables have the advantage of avoiding several statistical and inferential difficulties associated with multiple endpoints.

e. Dropouts

Dropouts are patients who, after a certain period of time in a trial, fail to provide clin efficacy data scheduled to be collected by the protocol. Frequently, dropouts occur reasons related to taking the assigned test drug (adverse effects or lack of efficacy) Since dropouts do not usually occur randomly, the remaining patients constitute a biased subsample of the patients originally randomized. Unless trial dropouts can b assumed to be random (which is rare), no model used to impute their effects can be verified.

Methods used to handle dropouts, such as LOCF and *completers* analyses, are not satisfactory even though they have often served as the basis for determining that adequate statistical evidence of efficacy has been provided. The LOCF method usu does not preserve the size of the test, either for the comparison of final observation for the comparison of rates of change. Alternative methods include growth curve analysis and random effects regression. These are also susceptible to informative censoring C that is, dropping out depends on the value of the response. It is often u to show that the results hold for a variety of analyses (i.e., they are robust).

f. Trials with several treatment groups/multiple comparisons

In clinical trials involving more than two treatment groups, a statistical multiple comparison procedure controlling the experiment-wise error rate to 5 percent or les should be applied. In essence, there should be overall statistical evidence of a treatment main effect before attempting to make specific drug comparisons relevan proposed drug labeling.

g. Trials simultaneously used to pursue more than one claim

A single trial can be used to pursue simultaneously more than one claim; an adjustr of significance level for multiple analyses is not always necessary. If the order of te the hypotheses is prespecified, then no penalty need be taken. For example, when trial is simultaneously pursuing a six-month signs and symptoms claim and a twelve month x-ray claim, if the trial wins by the first hypothesis tested C signs and sympto C then the x-ray hypothesis can be calculated without an adjustment penalty.

h. Interim analyses

Interim analyses are those that, for any purpose, are repeatedly performed on accumulating clinical trial efficacy data. Methods have been developed to compens for the fact that multiple tests (including interim analyses) alter the true significance level. The study protocol should state whether such interim analyses are planned o Should interim analyses be planned, the plan and its implementation should be described in the protocol. The description should include who will have access to th interim data, the scheduling of these interim analyses, the method to be applied for adjusting significance levels, and the corresponding time sequence of significance levels at which statistically significant results will be claimed.

Although an interim analysis may not be thought to affect the subsequent collection efficacy data, interim analyses carry the additional risk that the blinding or conduct study may have been compromised. Statistical methods cannot compensate for an unblinding and bias that may result from gathering the information needed to perfor interim analysis. Finally, if any major protocol change becomes necessary (e.g., a r therapy becomes available), it is important that such a change not be influenced by those unblinded to data.

i. Sample size

Failure to recruit an adequate number of patients is a major reason why an effectiv product may fail to meet established statistical criteria for efficacy, independent of whether the purpose was to show superiority or comparability of treatment effect. T method for determining the sample size should be stipulated in sufficient detail to p independent verification of the computation. This should include identifying the effic variable that the sample size determination is based on, the magnitude of the hypothesized clinical difference, the standard deviation, the power, the significance level, and the sidedness of the statistical procedure(s) described in the analysis pla Furthermore, the size of the clinical difference chosen should be justified, and the rationale for the choice of the efficacy variable used to determine sample size shou discussed. For comparability from one trial to the next, it is optimal to use the same efficacy variables as were used to power earlier studies.

j. Trials to show clinical equivalence

The words *clinical equivalence* are used in a much narrower sense than these word might imply to the casual reader. First, there is often no intent to show equivalence two or more drugs across the broad spectrum of pharmacologic effect. Rather, focu on showing no clinically relevant differences for one or possibly more variables that to be clearly identified in advance. The concept of equivalence is two-sided in that i any outcome measure, one drug is sufficiently different from another drug, then the drugs are no longer deemed equivalent in that variable.

To show equivalence, the variables serving to measure these effects of interest sh be defined in the protocol. For each efficacy variable for which clinical equivalence effect is sought, the magnitude of a difference deemed to be inconsequential shoul identified. The clinical data should then show, with 95 percent confidence, that this predefined difference is not exceeded.

Inference based on trials to show equivalence is inherently less convincing than inference based on trials to show the existence of a difference. Often, clinical trials not detect treatment differences that are known to exist. In such cases, statistical methods may then seemingly provide evidence of equivalent effect (e.g., to placeb

k. Appropriateness of the statistical methodology

The appropriateness of the statistical model should be assessed, including checkin outliers and determining if distributional assumptions (usually normality) are met an common variance assumptions hold

l. Site effects

If the patients have been stratified and randomized by site, the analysis should incl site effect. There may be a site-by-treatment interaction reflecting the degree to whi the treatment varies across sites. This is often notable when there is a great variati enrolled patients across sites. Site-by-treatment interaction should be explored.

F. Safety Analysis

The approach to evaluating adverse event data and laboratory values has traditionally differed fr that used to evaluate efficacy. The purpose of safety evaluations is usually not to test a specific hypothesis, but to examine the pattern of effects and to detect unusual or delayed events. Analys using cumulative occurrences, scatter-plots of laboratory values (baseline versus on-therapy), or other techniques may be helpful. The safety profile should address to what extent adverse event (drug reactions or lab values) depend on duration of drug exposure, dose level, coexisting medic conditions, or possible drug interactions. Incidence rates should be calculated using denominator that reflect the period of drug exposure for the population at risk. Cumulative incidences (hazard rates, instant probabilities) do a better job representing the temporal pattern of drug effects than prevalence rates; comparative cumulative incidence tables C drug versus active control(s), versu placebo C also are very helpful to practitioners. Critical incidence rates should be described with percent confidence intervals.

Ensuring safety during the development of a drug, biologic, or device can be optimized by adequ preclinical evaluations and the development of a standardized clinical safety assessment system Elements of a successful safety assessment system include the use of predefined standard terminology (such as the *Medical Dictionary for Regulatory Activities Terminology*) and criteria tc define and assess adverse events (AEs), approaches to optimize AE detection, and appropriate safety stopping rules in trials. It is also useful to capture AE severity (grades 1 [mild], 2 [moderate [severe], or 4 [life-threatening]), outcomes (such as the need for therapy and whether resolution death occurred), treating physician assessment of association with study agent (remotely, possib probably related), and impact on the trial (none, dose of agent delayed or changed, or patient withdrawn from further therapy). Stopping rules, determined by the risk/benefit ratio for the agent the study population, are desirable both for individual patients (a single grade 3-4 AE is often use as well as for the clinical trial, especially in dose-escalation studies.

1. Intrinsic trial design considerations

   An attempt should be made to characterize the patient population susceptible to adverse effects. Some extraneous factors, such as variations in soliciting and reporting adverse ev among the investigators and differences in the definition of normal ranges for lab values among different laboratories can complicate the safety data. Since adjustment for their eff may be difficult, precautions should be taken in the design stage of the trial to minimize th influence of these factors by preparing clear and specific instructions for data collection an monitoring adherence of the investigators and the laboratories to the protocol. Procedures normalizing laboratory data, for example, may be employed. As previously mentioned, developing standardized toxicity grading scales that can be employed in all studies may al be useful.

2. Adequate numbers

   The ability to detect adverse experiences is dependent on the number of patients evaluate the clinical trials and in clinical usage. In studies of 300 or more patients having adequate exposure to the investigational drug, it is expected (with 95 percent confidence) that at lea one patient will manifest an adverse event having an incidence rate of 1 percent or greate Smaller studies fail to meet even this minimal incidence detection standard. In most cases however, it is possible to combine studies of equal duration to establish adverse experienc rates.

   For any chronically administered product, the safety database should include at least 300 patients treated with the maximally recommended dose for at least six months and at leas patients treated for at least twelve months (ICH E1A 1995). Larger and/or longer safety databases may be advisable for agents with known or potential safety problems.

Table of Contents

IV. SPECIAL CONSIDERATIONS FOR BIOLOGICAL PRODUCTS 33

Although there are similarities between RA trial designs for drugs and biologics, biologics have special characteristics and problems that should be considered in their development.

A. Species Specificity

The schemes used traditionally in determining the initial human dose may not pertain to biologics Biological agents may behave differently in animal models than in humans, depending on the physiologic relevance and avidity for the receptor of the ligand in the animal compared to the hun Immunogenicity may also be species specific.

B. Dose Responses

The dose-response curve may be steep and/or even hyperbolic, and an agent can be quite toxic levels just above those thought to show efficacy.

C. Toxicity Response

The toxicity response may be highly unpredictable and potentially very dangerous and may inclu the risk of disease worsening. Agents may have narrow therapeutic windows. Biologics have the potential for disruption of immunologic and physiologic processes. Monoclonal antibodies to cellu epitopes of the immune system, for example, or to TNF receptors may cause serious morbidity a doses only slightly higher than those that are efficacious with markedly less toxicity.

D. Product Homogeneity

This often plays a critical role in activity and toxicity of a compound. Product alterations can greal affect physiologic activity. Thus, biologics should demonstrate lot-to-lot consistency to the extent possible while under development and be reasonably well characterized to be properly evaluatec

E. The Role of Antibodies

If phase 2 data suggest that agent-induced neutralizing antibodies could interfere with the efficac a biological agent over time, it may become necessary to formally investigate the possibility in a randomized-controlled setting. The occurrence of neutralizing antibodies may call for the reconsideration of doses and dose regimens. Non-neutralizing antibodies may have a profound e on PK and may therefore be just as important as neutralizing antibodies.

Table of Contents

V. SPECIAL CONSIDERATIONS FOR MEDICAL DEVICES

A. Background

Medical devices used in the treatment of RA vary considerably in their therapeutic impact, rangin from primary therapy for a specific affected joint to products used as adjuncts to primary therapie such as the use of ultrasound or heat for symptomatic relief in conjunction with pharmaceutical therapy. Variability in therapeutic effects due to disease and response heterogeneity may be exaggerated with some devices because treatments are often targeted to a single or a few joints patient.

Preclinical requirements for devices include assessment of data from a diverse range of tests, including, for example, tests that evaluate chemical composition, mechanical reliability or the electrical properties of a given device. Each product type has an identified battery of tests that ar evaluated to ensure that the design of the specific product is well understood and can be expecte

have the intended effect on patients as described in its labeling.

The design of clinical trials in RA for a device may raise some unique issues uncommon in pharmaceutical trials. The interaction between treated and untreated joints as it affects functional quality of life assessments is just one example of these issues. Selection of an appropriate contr often poses difficult questions when the active therapy under study is an implant and the availabl controls are more systemic in activity. Understanding how to design these trials, analyze the data and assess the contribution from each therapy can be challenging.

B. Efficacy Considerations

1. The selection of a control group may be quite challenging with devices because masking f the patient and investigator is frequently impossible and a placebo may be infeasible. Bec historical controls are often unsatisfactory and evaluating the equivalence of local therapy systemic active control poses problems, innovative trial designs are often needed. Some innovative options include randomization to early versus late device interventions, or resc interventions following failure of pharmaceutical interventions. Although the use of a sham (placebo) device is the most desirable control for many products evaluated for RA, it may l inappropriate if the subject device is implanted. Additionally, the success of patient and/or physician masking with such shams may not always be complete. Patient masking is infea if the product requires a surgical or other invasive procedure. However, in some cases it is possible to achieve evaluator masking for the effectiveness evaluation.

2. For devices intended for use as adjunctive therapy, approaches and analysis methods sh be designed to account for differences in disease status and severity to minimize potential biases in outcome measures. One approach is to have a consistent primary therapy with a named pharmaceutical to avoid confounding the analyses. The use of additional, potential confounding co-therapies (hot/cold treatments, splinting, physical therapy, and orthotics) a needs to be appropriately addressed during the study design phase.

3. The use of quality of life (QOL) assessments is very important where devices are intendec rehabilitative purposes. QOL benefits for the intervention should be judged with tools chos for their validity, ease, and convenience of administration and the ability to address both improvement in QOL and product satisfaction. These assessments should be kept maske from the independent effectiveness evaluator to avoid assessment bias.

4. In the case of invasive devices necessitating in-hospital or in-office use, it is recommende that clinical assessments include convenience in use and pain or discomfort in administrat Early assessment of this aspect of therapy can provide critical information on the ultimate acceptability of the treatment and affect decisions as to whether to pursue a treatment modality.

C. Safety Considerations

1. Obtaining well-characterized, short-term adverse events rates, as an early assessment of safety as is common for large pharmaceutical trials is not always meaningful in medical de trials. Device trials are frequently much smaller in size and the reliability of early data may be sufficient to establish a prospective pattern. When the device in question is an implant, evaluation period must be fairly long to assesses the potential for late occurring adverse events including device failure and adverse reactions to device materials. In some cases, full assessment of safety cannot be completed in the premarket phase and extends into mandatory postmarket surveillance.

2. When devices are used in conjunction with another medical or surgical procedure, the distinction between an adverse event that is clearly device related and one that is commor the procedure may be difficult to assess. The nature, timing, and degree of severity are so of the factors that may be useful in reaching a determination of cause. Since these determinations are based on clinical judgment, a real potential for bias exists in reaching conclusions about the treatment. Care must be exercised in designing the protocol to prov adequate detail in instruction to investigators to allow for consistent and unbiased decisior making on their part.

3. Devices (e.g., those emitting radiation) that have the potential for intermittent or chronic us require assessment as to whether there is a lifetime exposure limit or a maximum frequen exposure. Such issues are frequently addressed with animal data as well as with long-terr clinical evaluations.

Table of Contents

VI. SPECIAL CONSIDERATIONS FOR JUVENILE RHEUMATOID ARTHRITIS

A. Background

Juvenile rheumatoid arthritis (JRA) is a heterogeneous group of diseases that share the common feature of chronic, idiopathic synovitis, with onset prior to 16 years of age. These disorders have divided into clinically distinct subsets based on the extent of joint involvement and extra-articular manifestations: pauci-articular, poly-articular, and systemic-onset JRA, as well as oligoarthritis associated with HLA-B27,[4] and they have been further subdivided based on clinical courses (Ca: 1986). Immunogenetic subsets appear to correlate with these clinical course subsets and are als distinct from adult RA (Nepom 1991). Of these various entities, polyarticular JRA is similar in mar aspects to adult RA, particularly in clinical signs and symptoms, synovitis, and similar efficacy responses to some existing pharmacotherapy (NSAIDs, methotrexate, and prednisone). As only 5 percent of all patients with rheumatoid arthritis develop illness onset during childhood, many investigational therapeutic agents in this small population will receive orphan drug status, accordi to 21 CFR part 316 C Orphan Drugs. The application of principles in the conduct of clinical trials f adult RA largely applies as well to JRA, and this section outlines only those areas of difference fr adult RA. Sponsors are generally encouraged to develop as much information as possible on JR patients for agents that will be approved for adult RA. As a minimum, dosing and safety data are strongly encouraged.

Conducting drug studies in children is generally necessary and consistent with the expectations c treatment regimens for this disease. Because pediatric subjects constitute a vulnerable populatio conducting research involving minimal risk is important. The Committee on Drugs of the America Academy of Pediatrics has published guidelines for the ethical conduct of studies to evaluate dru pediatric populations (AAP 1995a), and general considerations for the clinical evaluation of drugs infants and children (AAP 1982), both of which should be consulted. Guidelines regarding inform consent and assent of pediatric patients from the Committee on Bioethics of the American Acade of Pediatrics should also be followed (AAP 1995b). Conducting clinical trials for patients with JRA and, particularly, assessing global disease activity and response to therapy should involve pediat rheumatologists or adult rheumatologists who have extensive training in pediatric rheumatology a have demonstrated competence in caring for children with rheumatic diseases.

As a general principle, children should not be subjected to an agent that has not been first tested safety in adults. Testing may begin in children, however, when the anticipated benefits based on existing knowledge justify the anticipated risks. An agent developed specifically for use in JRA (e biological agent targeted against a specific pathogenic process that is unique to JRA and not pre in adult RA) may need to be tested first in children, as exposure in adult RA patients or even norr adult volunteers may be unrevealing. If, however, the agent has potential for use in both adult RA JRA, then, at minimum, PK-PD and initial phase 1 data (including MTD) for adults should be avai prior to the start of testing in children. JRA trials of drugs that are expected to be similar in efficac existing drugs and that do not represent major therapeutic advances or alternative approaches to basic mechanism of intervention can be delayed until there is extensive efficacy and safety data either from adults or in other pediatric populations.

The need for reliable inferences does not necessitate a placebo control, but randomization and controls should be employed. The choice of control is a function of what is known about the agen the time and what other treatments are available to potential trial enrollees. If only an active contr used for an equivalence trial, convincing evidence of the efficacy of the active control should be provided, and the test proposed to establish equivalence should be specified. If there have been prior adult studies, or if the agent under development has a novel mechanism of action or repres an entirely new class of drug, a randomized, double-blinded trial, using either a placebo or an act control group of (anticipated) similar efficacy is indicated. Open label extensions to obtain additio data about risk and persistence of benefit are very valuable. The use of active control (standard-

care therapy) in the control arm, dose-response design (where control receives a lower dose(s) c test agent), crossover, randomized withdrawal (enrichment design) or, if the agent has a short on of effect, randomized placebo-phase trial designs are encouraged as possible alternatives to inac placebo control in JRA studies (Temple 1994, Feldman 1995). As a general principle, protocol es clauses are encouraged to permit children who are not responding well to experimental therapy t receive early conventional or alternative treatment. The sponsor should indicate how dropouts wi handled in the analysis, whether from the escape clause, or otherwise.

B. Applicability of Pediatric Regulation and Impact on Trial Design for JRA Studies

The *pediatric use* section in the labeling regulations (21 CFR 201.57(f)(9)) permits drug and biolo products to be labeled for pediatric use if they have been demonstrated to be safe and effective f adult populations and the mechanism of action of the drug is sufficiently similar in children. The pediatric rule may be applied only to obtain labeling for the signs and symptoms of JRA; other cla including prevention of structural damage, remission, and prevention of disability, should be evaluated in separate JRA efficacy studies. Although the regulation allows extrapolation of adult efficacy data, additional pediatric dosing and safety evaluations are usually needed.

In general, sponsors seeking approval for adult RA products appropriate for use in patients with are strongly encouraged to obtain dosing and safety data in polyarticular course JRA for inclusion the dosing and pediatric use sections of the label. Specimen collection for PK studies can be redu significantly if available data indicate that the coefficients are similar in adults and children. Microsampling techniques should be employed for such studies (Hashimoto 1991). The extent of safety testing needed depends on the agent, its prior use, and any established safety in other pediatric populations. Toxicity grading scales should be adjusted for pediatric populations. Phase studies for safety evaluation will be strongly encouraged when limited preapproval data are obtai It is desirable that as much efficacy evidence as possible be gathered during the evaluation of pediatric dosing and safety.

For currently approved traditional (cyclooxygenase inhibitor) NSAIDs and corticosteroids, adequa efficacy information exists to support a labeled indication for all JRA and all JRA subsets. For methotrexate and sulfasalazine, adequate efficacy information exists for a labeled indication for J patients with a polyarticular course. For such agents, a labeling claim could be supported using c PK, PD, and safety data in JRA patients, although submission of additional JRA efficacy data is encouraged.

For new agents (not yet approved for adult RA) that are not from a new pharmacologic class, adu efficacy data can be used to support a signs and symptoms claim for polyarticular JRA if there is biological plausibility that the agent would have a similar effect in JRA. The applicability of the pediatric rule to support a labeled indication for polyarticular course JRA will be based on adult R efficacy data considered on an individual basis for each agent. When evidence for biological plausibility does not exist, evidence should be submitted to support the application of the pediatri rule. (The Agency should be consulted in determining whether adequate biological plausibility exi to apply the pediatric rule.) Pediatric safety and dosing studies should be submitted.

For agents in a new class, efficacy studies should be performed in JRA to obtain an indication for in JRA. The indication will reflect the JRA subsets included in the efficacy study. Sponsors who s approval for all JRA should include all JRA subsets in an efficacy study. The data could support a claim for JRA (subsets not specified) provided that the data do not suggest that the agent is ineffective in any one subset. The label should reflect that efficacy was demonstrated and that th agent is approved for JRA (subsets specified depending on which were included in the efficacy study).

C. Outcome Variables and Claims

It is possible for sponsors to seek approval for all JRA subsets or to seek approval for individual subsets. In the former case, the label should note the number of patients from each subset enroll trials and the character of each subset response. Except as noted above in the application of the pediatric rule, all claims should be supported by an efficacy demonstration in the intended subset

1. Reduction in the Signs and Symptoms of JRA

All JRA trials should evaluate improvement based on a validated endpoint for improvemer Currently, the one validated approach is the definition of improvement established by the core set: three of six (MD global, parent/patient global, number of active joints, number of with limited range of motion, functional ability, and ESR) improved by at least 30 percent a no more than one of six worsening by more than 30 percent (Giannini 1997). Because the definition of improvement was validated using a trial of methotrexate, which primarily inclu polyarticular JRA patients, protocol individualization may necessitate a refinement in the responder test for other patient subsets. For example, for pauci-articular JRA, with one kn involved and a normal ESR, use of joint and functional assessments specific to the involve joints and evaluation of uveitis as coprimary endpoints may also be valuable (Lindsley 19 For patients with systemic onset JRA, additional assessment of fever, extra-articular manifestations, and thrombocytosis/leucocytosis may be useful coprimary endpoints (Silverman 1994). Outcome variables need to be appropriate and consistent with the type agent under investigation. Investigators should specify, before the trial is initiated, how mu change is considered clinically important for each outcome variable.

Trials should generally last at least six months, except when six-month efficacy data exist adult RA and there are no reasons to expect loss of efficacy over time. Under these circumstances, trial durations may be three months' blinded/randomized, but six-months' c safety data should be obtained. As with adult RA, a three-month trial duration is suggested NSAIDs.

2. Major Clinical Response

Similar to adult RA, major clinical response is a claim intended to connote that the agent provides substantial clinical benefit, including in patients who are unable to completely res to the treatment or remit from the disease. At present, this claim is only theoretical, as clini JRA trial databases adequate for defining major clinical response do not exist.

3. Complete Clinical Response

The claim of complete clinical response reflects achievement on drug of six consecutive months of morning stiffness of less than 15 minutes duration, no active synovitis (pain, redness, tenderness to palpation, swelling, stable or decreasing limitation of motion), no e articular features (including fever, serositis, adenopathy, hepatosplenomegaly, rash, uveiti and normal laboratory parameters (including ESR, platelets, WBC) and, where applicable, ongoing structural damage while continuing on therapy. Trials should be at least one year duration. Residual damage from prior disease, including extra articular manifestations, is acceptable in meeting criteria for complete clinical response. Because spontaneous comp clinical response rates may be relatively high in JRA, these studies should be controlled.

4. Remission

Remission is characterized exactly as above, but while off all antirheumatic drugs. Remiss is not intended to imply cure.

5. Prevention of Disability

This claim is proposed to reflect durable improvement in physical function and disability in studies of one to two years' duration with demonstrated improvement in signs and sympto over the same period. Instruments currently validated for use in JRA include the Childhoo Health Assessment Questionnaire (CHAQ), the Juvenile Arthritis Self-Report Index (JASI) the Juvenile Arthritis Functional Assessment Report (JAFAR). HR-QOL should also be measured and demonstrated not to worsen over the trial duration. Endpoints should be tai to subtypes enrolled in trials (e.g., to assess knee function in pauci-articular JRA patients i whom knee arthritis may be the primary arthritic manifestation). Instruments should be developmentally validated for the age ranges studied in a trial (Murray 1995).

6. Prevention of Structural Damage

Similar to adult RA, this claim would reflect trials of one year or more with concomitant suc in signs and symptoms. Currently, only sparse data exist regarding the usefulness of only

radiographic measure in JRA: the carpal-metacarpal distance in those patients with wrist arthritis. Other clinically promising settings include the evaluation of erosive disease in systemics with polyarthritis, hip assessment in systemics, and knee assessments in pauci-articular JRA.

D. Trial Design Issues

Recommendations for efficacy studies are based on the nature of the agent under development. principles outlined for adult RA are generally applicable. Patients enrolled in these trials may be c any onset or disease course subset. Separate trials for each JRA subset are recommended if the agent is predicted to have a target mechanism of action that will not be applicable and equally efficacious in all JRA subsets. Alternatively, a single, sufficiently large trial with enrollment appropriately stratified provides for useful conclusions to be reached about efficacy and safety fo each subset. Relevant covariates include disease course type, disease duration, and nonrespons prior methotrexate treatment. Given that JRA is an orphan disease, there is often some flexibility trial design, but this should be discussed on a case-by-case basis.

At this time, JRA patients are usually ineligible for entry into efficacy trials unless they have failed respond adequately to at least one standard second line agent (such as methotrexate at a dose c least 10 mg/m2 body surface area per week). There may be exceptions to this if, for example, the evidence that greater efficacy could be obtained by using the agent very early in the disease cou evidence that delayed use in sicker patients potentially carries greater risk of toxicity, or evidence the agent has a favorable safety and efficacy profile in a comparable population studied to date a that the agent's actions are potentially readily reversible. Pauci-articular JRA patients are particul encouraged for inclusion in trials with agents targeting the treatment of uveitis or agents that will replace existing therapy with an improved safety profile, less frequent blood monitoring, and/or superior efficacy.

Some JRA patients exposed to new agents should be evaluated periodically for an extended peri Effects on skeletal growth, development, behavior, sexual maturation, reproductive capacity, and secondary malignancy should be included in such monitoring. Registries or cohort follow-up stud may be useful in providing long-term safety information.

E. Concurrent Antirheumatic Agent Administration

The principles of use of concurrent antirheumatic therapy in JRA trials are similar to those outline adult RA: limiting their discretionary use as much as reasonably possible so that interpretation of efficacy and safety data is not compromised. However, limitations on concurrent medication cann prohibit ethically justified treatments, nor should the protocol be made so unattractive to parents, physicians, and patients that enrollment is threatened. If background treatment is necessary, earl tolerance studies, to ensure safety of co-administration, should precede any large trials.

If patients receive concurrent slow-acting or prednisone therapy, the dose should be stable prior study entry and should preferably remain so throughout the trial. Concurrent medications are usu important prognostically and so may need stratification. If possible, intra-articular steroid injection should be disallowed for a minimum of one month prior to beginning experimental therapy; othen that joint should be discounted in assessing therapeutic effects.

F. Multicenter Trials and Center Effects

Although JRA is the most common rheumatic disease of childhood, its prevalence is low compar adult RA. Thus, trials of JRA that require large numbers of patients will likely be multicenter trials Multicenter trials should employ a standardized protocol and data collection forms among all cen Pretrial meetings of all investigators and other involved personnel are strongly encouraged to ens uniformity in protocol interpretation, patient evaluation, and data recording. Studies have shown t within a cooperative group, a center's performance is a function of the number of patients enrolle the center (Sylvester 1981). Thus, studies that use fewer centers with greater numbers of patient each center are preferable to those that use large numbers of centers with fewer patients. Effort should be made to enroll at least 10 to 12 patients at each center to provide for greater quality assurance. In all multicenter trials, center effects should be examined. A therapy should show eff in more than one center. When stringent entrance criteria restrict the number of patients eligible f study, many centers may be unable to enroll even 10 patients. In such situations, randomization blocked within individual centers, rather than across all centers, may help to reduce the potential

impact of center effects.

Table of Contents

REFERENCES

American Academy of Pediatrics (AAP), Committee on Drugs, 1982, *General Considerations for the Clinical Evaluation of Drugs in Infants and Children,* Report to the U.S. Food and Drug Administration, available throug Freedom of Information Office of the FDA, FDA Contract Nos. 223-79-3003 & 223-82-3009.

_____, Committee on Drugs, 1995a, "Guidelines for the Ethical Conduct of Studies to Evaluate Drugs in Pedia Populations," *Pediatrics,* 95: 286-294.

_____, Committee on Bioethics, 1995b, "Informed Consent, Parental Permission, and Assent in Pediatric Prac *Pediatrics,* 95: 314-317.

Cassidy, J.T., J.E. Levinson, J.C. Bass, et al., 1986, "A Study of Classification Criteria for a Diagnosis of Juver Rheumatoid Arthritis," *Arthritis and Rheumatism,* 29: 274-281.

Feldman, B.M. and J.P., Szalai, 1995, "Towards a More Ethical Clinical Trial Design: The Randomized Placeb Phase Design (RPPD)," abstract, *Arthritis Rheum,* 38 (supplement): s177.

Felson, D.T., et al., 1993, "The American College of Rheumatology Preliminary Core Set of Disease Activity Measures for Rheumatoid Arthritis Clinical Trials," *Arthritis and Rheumatism,* 36(6): 729-740.

Felson, D.T., et al., 1995, "American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis," *Arthritis and Rheumatism,* 38(40): 1-9.

Food and Drug Administration, 1978, *General Considerations for the Clinical Evaluation of Drugs,* FDA 77-304

_____, 1988, *Guidelines for the Clinical Evaluation of Anti-Inflammatory and Antirheumatic Drugs (Adults and Children).* April 1988.

_____, 1997, *Guidance for Industry: Drug Metabolism/Drug Interaction Studies in the Drug Development Proc Studies In Vitro,* April 1987.

_____, 1993, "Guideline for the Study and Evaluation of Gender Differences in the Clinical Evaluation of Drugs *Federal Register,* July 22, 1993, 58 (139): 39406-39416.

Fries, J.F., P.W. Spitz, and D.Y. Young, 1982, "The Dimensions of Health Outcomes: the Health Assessment Questionnaire, Disability, and Pain Scales," *Journal of Rheumatology,* 9: 789-793.

Giannini, E.H., N. Ruperto, A. Ravelli, et al., 1997, "Preliminary Definition of Improvement in Juvenile Arthritis," *Arthritis and Rheumatism,* 40: 1202-1209.

Hare, L., L. Wagner-Weider, A. Poznanski, et al., 1993, "Effects of Methotrexate on Radiologic Progression in Juvenile Rheumatoid Arthritis," *Arthritis and Rheumatism,* 36: 1370-1374.

Hashimoto, Y., and L.B. Sheiner, 1991, "Designs for Population Pharmacodynamics: Value of Pharmacokineti Data and Population Analysis," *Journal of Pharmacokinetics and Biopharmaceuticals,* June 1991, 19(3): 333-5 NLM CIT. ID: 91341632.

ICH, "S5A Guideline on Detection of Toxicity to Reproduction for Medicinal Products, "Federal Register, Thurs September 22, 1994, 59 (183): 48746-48752.

_____,"S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals," *Federal Register,* April 4, 1997, 62 (65): 16437-16442.

_____,"EIA Extent of Population Exposure Required to Assess Clinical Safety for Drugs Intended for Long-Ter Treatment of Non-Life-Threatening Conditions," *Federal Register*, March 1, 1995, 60 (40): 11269-11271.

Larsen, A., K. Dale, and M. Eek, 1977, "Radiographic Evaluation of Rheumatoid Arthritis and Related Conditio by Standard Reference Films," *Acta Radiol. Diag.* 18: 481-491.

Lindsley, C., 1996, "Juvenile Rheumatoid Arthritis Workshop," Proceedings, pp. 34-43.

Little, R., and D. Rubin, 1987, Statistical Analysis with Missing Data, John Wiley & Sons.

Meenan, R.F., P.M. Gertman, J.H. Mason, et al., 1982, "The Arthritis Impact Measurement Scales: Further Investigations of Health Status Measure," *Arthritis and Rheumatology,* 25: 1048-1053.

Murray, K.J., and M.H. Passo, 1995, "Functional Measures in Children with Rheumatic Diseases," *Pediatric Cl of North America,* 42: 1127-1154.

Nepom, B., 1991, "The Immunogenetics of Juvenile Rheumatoid Arthritis," *Rheumatic Disease Clinics of North America,* 17: 825-842.

Paulus, H.E., M.J. Egger, J.R. Ward, et al., 1990, "Analysis of Improvement in Individual Rheumatoid Arthritis Patients Treated with Disease-Modifying Antirheumatic Drugs Based on Findings in Patients Treated with Placebo," *Arthritis and Rheumatism,* 33: 477-484.

Pinals, R.S., et al., 1981, "Preliminary Criteria for Clinical Remission in Rheumatoid Arthritis," *Arthritis and Rheumatism,* 24 (10): 1308-1315.

Sharp, J.T., D.Y. Young, G.B. Bluhm, et al., 1985, "How Many Joints in the Hands and Wrists Should Be Incluc in a Score of Radiologic Abnormalities Used to Assess Rheumatoid Arthritis?" *Arthritis and Rheumatism,* 28: 1 1335.

Silverman, E., G.D. Cawkwell, et al., 1994, "IVIG in the Treatment of Systemic Onset JRA," The *Journal of Rheumatology,* 21(12): 2353-2358.

Smolen, J.S., F.C. Breedveld, G. Eberl, et al., 1995, "Validity and Reliability of the Twenty-Eight-Joint Count fo Assessment of Rheumatoid Arthritis Activity," *Arthritis and Rheumatism,* 38: 38-43.

Sylvester, R.J., H.M. Pinedo, M. De Pauw, et al., 1981, "Quality of Institutional Participation in Multi Center Clir Trials," *New England Journal of Medicine,* 305: 852-855.

Temple, R.J., 1994, "Special Study Designs: Early Escape, Enrichment, Studies in Non-Responders." *Comm. Statistic. Theory Meth.* vol 23(2): 499-531.

Table of Contents

APPENDIX A: COMPARATIVE TRIAL RESPONSE RATES[5]

Three cooperative systematic studies of rheumatic diseases (CSSRD) trials:
(1) Methotrexate vs. Placebo
(2) Gold, Auranofin vs. Placebo
(3) D-Penicillamine high, low vs. Placebo

Response Rates at End of Trial Based on Different Definitions of Improvement

---

Definition of Improvement	PLACEBO n=199	(Auranofin) (Low-dose D-Penicillamine) n=18	High-dose D-Penicillamine Gold, Methotrexate n=155
ACR≥ 20%	10 (8.4%)	30 (25.4%)	64 (40.3%)
ACR≥ 30%	.5 (4.2%)	14 (12.0%)	46 (29.7%)
ACR≥ 40%	2 (1.7%)	7 (3.4%)	18 (11.6%)
ACR≥ 50%	0 (0%)	4 (3.4%)	14 (9.0%)
ACR≥ 60%	0 (0%)	3 (2.5%)	4 (2.6%)
ACR≥ 70%	0 (0%)	0 (0%)	1 (0.6%)

Table of Contents

APPENDIX A (cont.)

COMPARATIVE MULTICENTER TRIAL OF AURANOFIN/METHOTREXATE (END OF TRIAL)

Response Rates at End of Trial Based on Different Definitions of Improvement

Definition of Improvement	Auranofin (n=118)	Methotrexate (n=119)
ACR≥ 20%	34 (28.8%)	77 (64.7%)
ACR≥ 30%	30 (25.4%)	65 (54.6%)
ACR≥ 40%	22 (18.6%)	51 (42.9%)
ACR≥ 50%	21 (17.8%)	42 (35.3%)
ACR≥ 60%	9 (7.6%)	22 (18.5%)
ACR≥ 70%	7 (5.9%)	11 (9.2%)

Table of Contents

APPENDIX A (cont.)

COMPARATIVE TRIAL OF CYCLOSPORINE-A METHOTREXATE VS. METHOTREXATE ALONE

Response Rates at End of Trial Based on Different Definitions of Improvement

Percent Increase by ACR Criteria	Patients Satisfying Criteria

	Methotrexate+ Cyclosporine-A (n=71)	Methotrexate+ Placebo (n=74)
0%	81.7	50.0
10%	49.3	16.2
20%	45.0	12.2
30%	33.8	8.1
40%	22.5	2.7
50%	22.5	2.7
60%	5.6	2.7
70%	1.4	0.0
80%	0.0	0.0
90%	0.0	0.0

Table of Contents

---

[1] This guidance has been prepared by the Rheumatology Working Group of the Medical Policy Coordinating Committee (MPCC) of the Center for Evaluation and Research (CDER), the Center for Biologics Evaluation and Research (CBER), and the Center for Devices and Radiological Health (CDRH).

[2] The ACR definition of Improvement (ACR 20) is 20 percent improvement in tender and swollen joint counts and 20 percent improvement in three five remaining core set measures: patient and physician globals, pain, disability, and an acute phase reactant (Felson 1993, 1995). For specific de on individual measures, e.g., disability, acute phase reactant, refer to Felson 1995.

[3] ICH documents are available via the FDA Internet home page at http://www.fda.gov/cder or cber.

[4] The HLA-B27 subset is not addressed in this document.

[5] CSSRD database and Tugwell et al., "Combination Therapy with Cyclosporine and Methotrexate in Severe Rheumatoid Arthritis," N Engl J Med 333;137-141, 1995.

Table of Contents

Last Updated: 4/16/2002

EXHIBIT 8

AUSTRALIA

Patents Act 1990

IN THE MATTER OF
US Patent Application No. 09/446,109
by The University of Queensland

EXHIBIT SMT-8

This is Exhibit SMT-8 referred to in the Statutory Declaration by Stephen Maxwell Taylor

dated 12 MAY 2004

Before me:

Toni Vuo

JUSTICE OF THE PEACE (QUALIFIED)
DEPT OF JUSTICE & ATTORNEY-GENERAL
PRO. NO. 82333

A person empowered to witness Statutory Declarations under the laws of the Queensland, Commonwealth of Australia

PRM-01-03

Open Label Safety and Tolerability Study of Topical PMX53 in Subjects with Psoriasis

Psoriasis Trial

- Primary Objective
  - Evaluate the safety and tolerability of topically administered PMX53 twice daily for 56 days to target lesions of subjects with psoriasis

- Secondary Objective
  - Evaluate the effect of topical administration of PMX53 on disease status of target lesion

Psoriasis Trial

- Single dose application in healthy volunteers
  - 3 subjects, single application
- Multiple dose application in healthy volunteers
  - 3 subjects, twice daily 4 days
- Multiple dose application in psoriasis patients
  - PMX53 Gel (10mg/ml) will be applied to target lesion twice daily for 56 days

Psoriasis Trial

- 10 subjects (for main part of study)
- Mild to moderate chronic plaque type psoriasis, for at least one year
- Target lesion must have
  - Severity Index score (LPSI) of 5-8
  - Area of 10 - 100cm^2;
  - Stable in both extent and severity for two weeks prior to treatment

  Definition LPSI: summed score for
  - erythema, induration and desquamation of target lesion,
  - scale of 0 – 12
  - higher score = more severe disease
  - decrease in LPSI score = improvement

Psoriasis Trial

Safety and Tolerability

- No Serious Adverse Effects
- 16 AE's reported
- Ranging mild to severe (one subject had severe cold and sore throat)
- All classified as not related or unlikely to be related
- Include back pain, headache, sinusitis, head cold, common cold, inflamed psoriasis (1 subject)

Psoriasis Trial

Disease Assessment

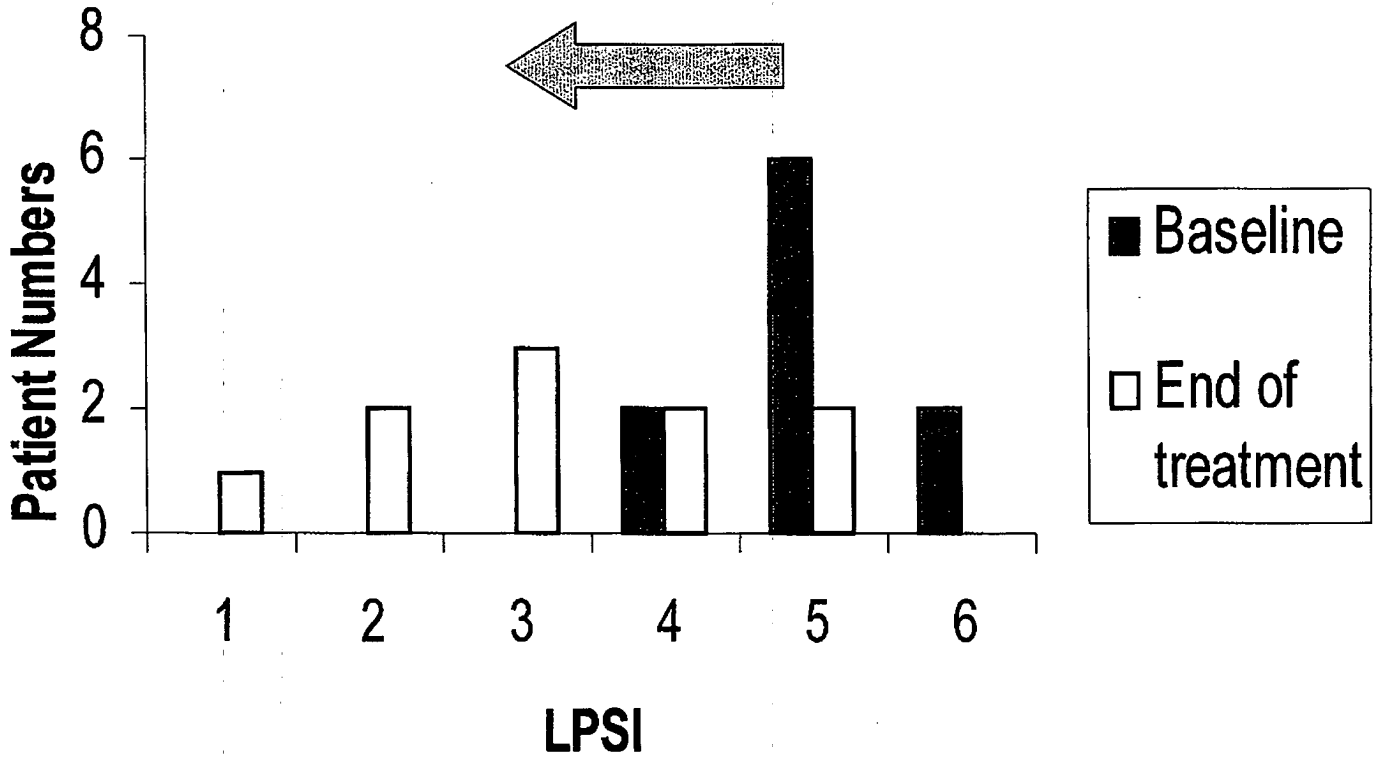
- 9/10 patients had improved LPSI score by the end of study

- 1 patient showed improvement of 4 points,
- 4 patients improved by 2 points, and
- 3 patients improved by 1 point

- 8 patients reported improvement in their subjective assessment of the psoriatic lesion

Psoriasis LPSI scores

Lower LPSI scores indicate improvement



Psoriasis Trial Summary

- PMX53 Gel (10mg/ml) is safe and tolerable over 56 days
- Data indicates a moderate clinical response

RA Trial Interim Analysis

A double-blind study evaluating the safety of PMX53 in comparison to placebo in patients with active rheumatoid arthritis

RA Trial Objectives

Primary objective:

Evaluate the safety and tolerability over 28 days dosing *(recall Phase Ia single dose safety study)*

Secondary objective:

Assess pharmacokinetics of PMX53 in acute and chronic dose setting

Assess biological activity

- synovial biopsy tissue assessment (only at end of study)
- Biochemical markers - CRP, ESR,
- Standard disease measures
- Physician assessment of disease
- Patient self assessment of disease, health and pain

RA Trial Protocol

- Randomised, double blind and placebo controlled
- 10 patients with active rheumatoid arthritis on methotrexate for 3 months and a stable dose (5-30 mg/week) for at least one month

  Active disease defined as
  = 6 tender and = 6 swollen joints AND ESR = 28mm/hr
  or CRP = 10mg/L
  or morning stiffness = 45 minutes

- Daily oral dose of 8 mg/kg for 28 days *(recall Phase Ia max single dose 10 mg/kg)*
- Safety evaluation: continuous throughout study
- PK profile Day 1 & Day 27 – trough levels days 7, 14

PMX53 was Safe and Tolerable

- **PMX53 group (n = 7)**
  - Total No. AEs = 9 across all patients (3 patients no AE's)
  - 2 patients had "moderate" severity AEs - both "unlikely" relationship
  - 4 patients had 7 "mild" severity AEs, 1/7 classed as "possibly" related

- **PLACEBO group (n = 3)**
  - Total No. AEs = 13
  - All mild, 6/13 classed as "possibly" related to drug

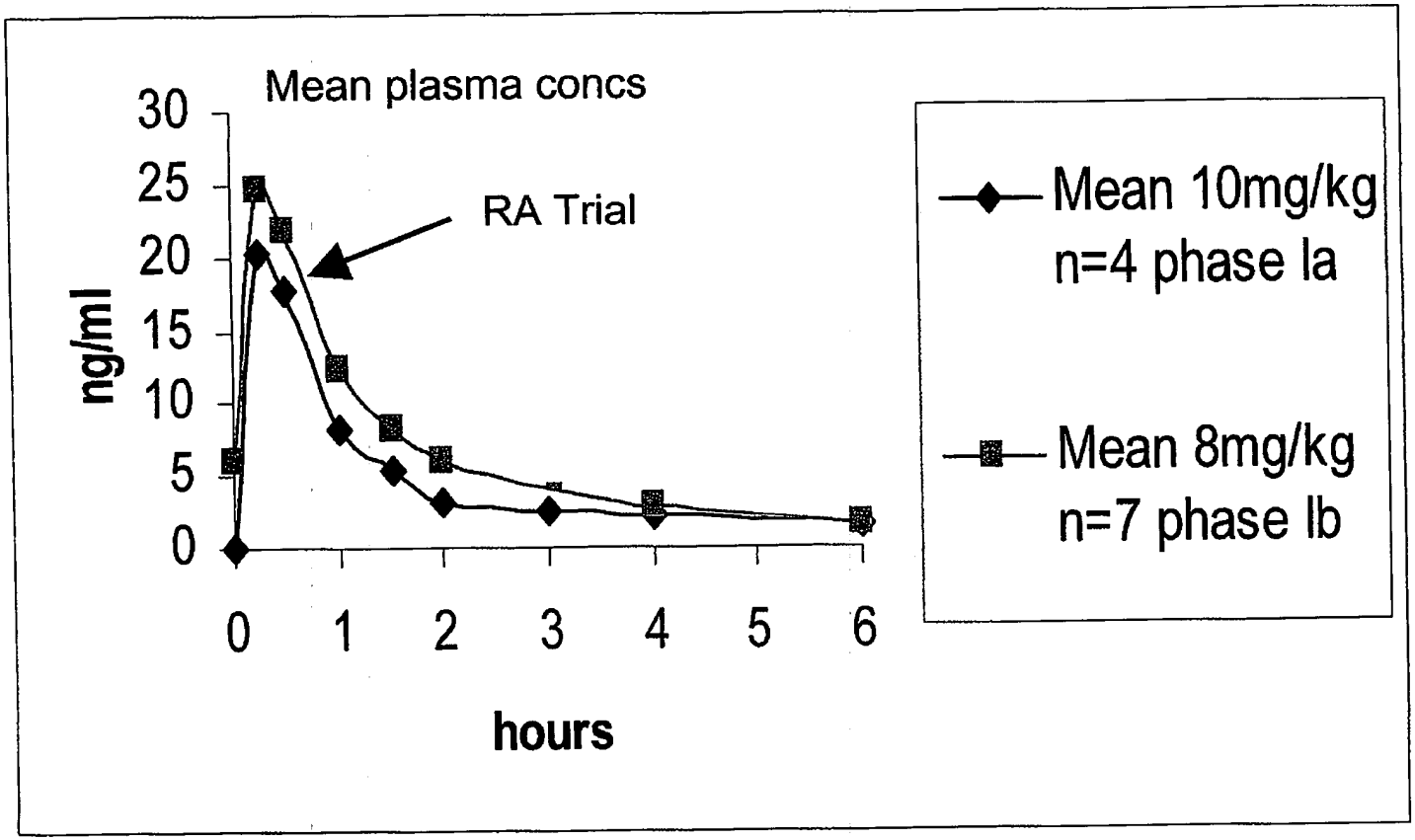
* Primary Objective of Study likely to be achieved

PMX 53 Pharmacokinetics

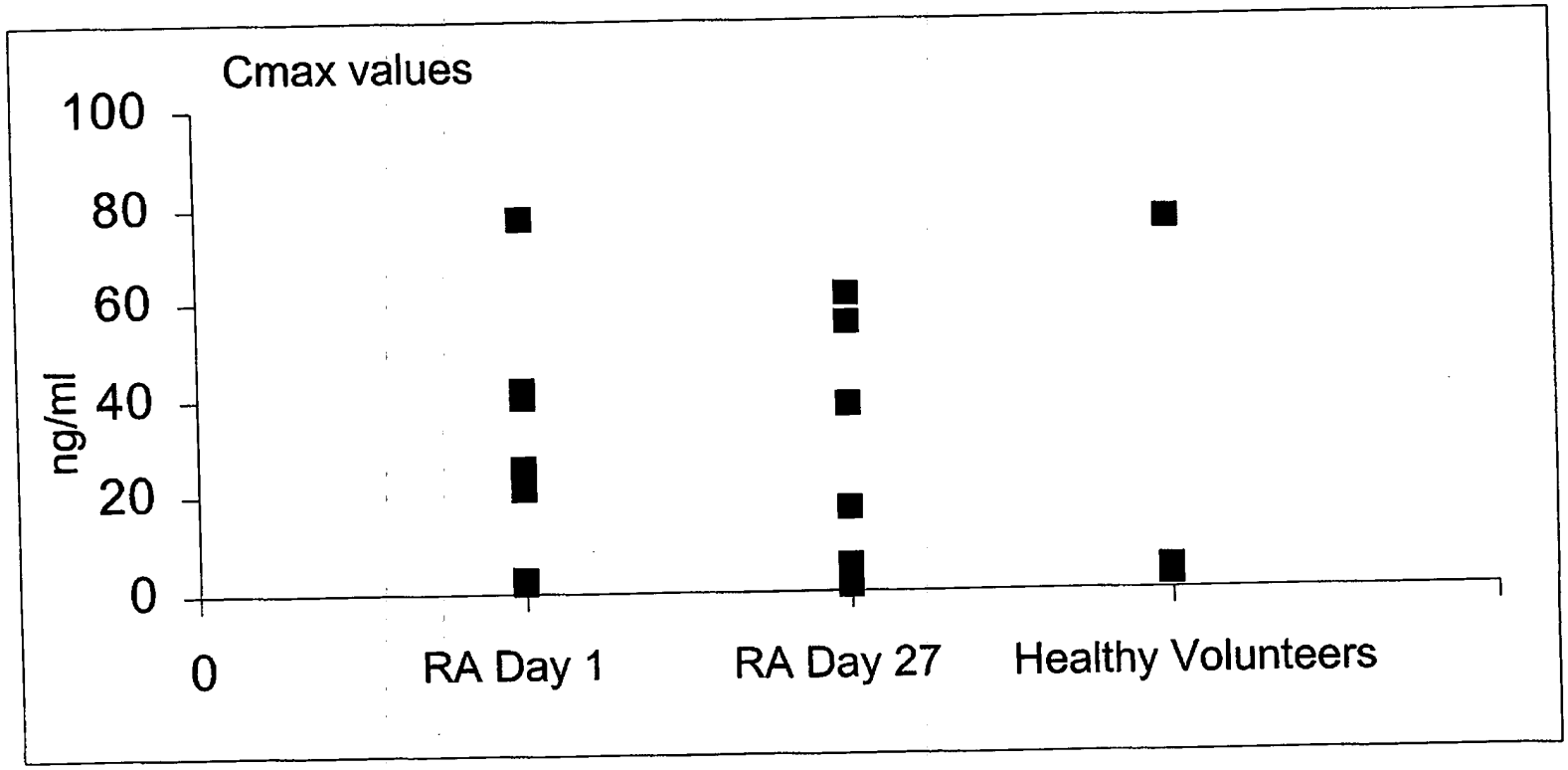
- PK profiles similar to that seen in healthy volunteers
- Blood levels typically higher
- Observe same wide variation between subjects with Cmax ranging from 1 – 40 ng/ml
- No accumulation seen with chronic dosing

PMX 53 Pharmacokinetics

PK Profiles similar across studies



PMX 53 Pharmacokinetics
Range Cmax values



Wide interpatient variability / intrapatient consistent

RA Trial Disease Measures

- Patients with elevated baseline CRP
  - 4/4 subjects on PMX53 showed decrease
  - 1/1 subjects on placebo showed increase
- ESR
  - No change observed
- Clinical
  - No trends observed in tender and swollen joints

RA Trial Disease Measures

- Patient Assessment
  - Patient Global Assessment of disease (VAS),
    - PMX 53 all patients showed an increase in assessment with 2 showing = 10 points improvement
    - All placebos showed worsening of at least 10 points
  - Patient Assessment of Health (VAS)
    - PMX53 recorded improvement in 3/7 patients
    - Placebo recorded worsening in 3/3 patients
  - VAS pain score
    - PMX53 3/7 (42%) less pain 4/7 unchanged
    - Placebo 1/3 (33%) less pain 2/3 worsened
- Physician Assessment (VAS)
    - PMX53 3/7 (42%) improved and 4/7 unchanged
    - Placebo 1/3 (33%) improved 2/3 worsened

RA Trial Sub-study

- Ex-vivo Blood Study ("Reedquist Study")

  Analysis of Leukocyte Function and Survival
  - Blood samples taken from all patients at
    - Baseline, post 6hr, trough day 7, 14 and Day 27
  - Large inter- and intra-patient variation in control data observed in placebo treated patients
  - Against this background variability it is impossible to interpret data

RA Trial Interim Results Summary

- No safety issues to hinder continuation of study
- Highly likely to reach primary endpoint of safe and tolerable dosing over 28 days
- PK profile replicates phase I healthy volunteer study
  - Increased frequency of higher blood plasma levels
  - Large interpatient variation
  - Low intrapatient variation
- Some disease assessments showing moderate positive trends
- Ex-vivo blood analysis disappointing due to technical issues
- Key biological markers from synovial tissue biopsy to be analyzed at end of study

RA Trial Progress

- A total of 14 subjects have completed treatment and 1 is currently on study
- A further 4 patients identified for study and awaiting prescreening
- Aiming to recruit a total of at least 20 subjects as fast as possible

ALTERNATE OPTION cont…

- Subcutaneous dosing with PMX53

If Phase I s.c study positive

- Provides licensee clear path for development
- Takes oral delivery form off critical path

Downside

limit potential clinical use to short term indications eg IBD relapse, treatment of acute inflammatory episodes

ALTERNATE OPTION…

- Subcutaneous dosing with PMX53
  - subcutaneous preclinical data shows sustained blood levels over many hours
  - Preclinical rats and dogs data demonstrate efficacy at dose levels of 0.3 mg/kg
    - Positive for COGS issue
  - Work up to human phase I study to show:
    - Sustained blood levels
      .. addresses "bioavailability/transient" PK issue
    - If available assess also using biomarker assessment

PLAN GOING FORWARD…

Continue to develop Biomarker for PMX53

- Option 1 Oxidative Burst Assay
  - Questions regarding sufficient sensitivity to detect activity?
  - Inter- and Intra-assay data adequate to show effect?
  - Assay work ongoing at Promics labs
- Option 2 LPS Model
  - Clinical trials using LPS challenge have been conducted
  - Requires validation in preclinical before assessing healthy volunteer study
  - Ongoing assessment at Promics labs

Two major challenges remain…

- Demonstration of adequate and sustained block of C5a receptors with PMX53 in face of transient PK profile
  - If +ve disease efficacy....resolved v
  - If disease efficacy equivocal ....need biomarker data
- Estimation of Cost of Goods
  - Highly dependent on dose required
  - Use biomarker to estimate efficacious doses required

RA Trial Progress

- A total of 14 subjects have completed treatment and 1 is currently on study
- A further 4 patients identified for study and awaiting prescreening
- Aiming to recruit a total of at least 20 subjects as fast as possible

RA Trial Interim Results Summary

- No safety issues to hinder continuation of study
- Highly likely to reach primary endpoint of safe and tolerable dosing over 28 days
- PK profile replicates phase I healthy volunteer study
  - Increased frequency of higher blood plasma levels
  - Large interpatient variation
  - Low intrapatient variation
- Some disease assessments showing moderate positive trends
- Ex-vivo blood analysis disappointing due to technical issues
- Key biological markers from synovial tissue biopsy to be analyzed at end of study

RA Trial Sub-study

- Ex-vivo Blood Study ("Reedquist Study")

  Analysis of Leukocyte Function and Survival
  - Blood samples taken from all patients at
    - Baseline, post 6hr, trough day 7, 14 and Day 27
  - Large inter- and intra-patient variation in control data observed in placebo treated patients
  - Against this background variability it is impossible to interpret data

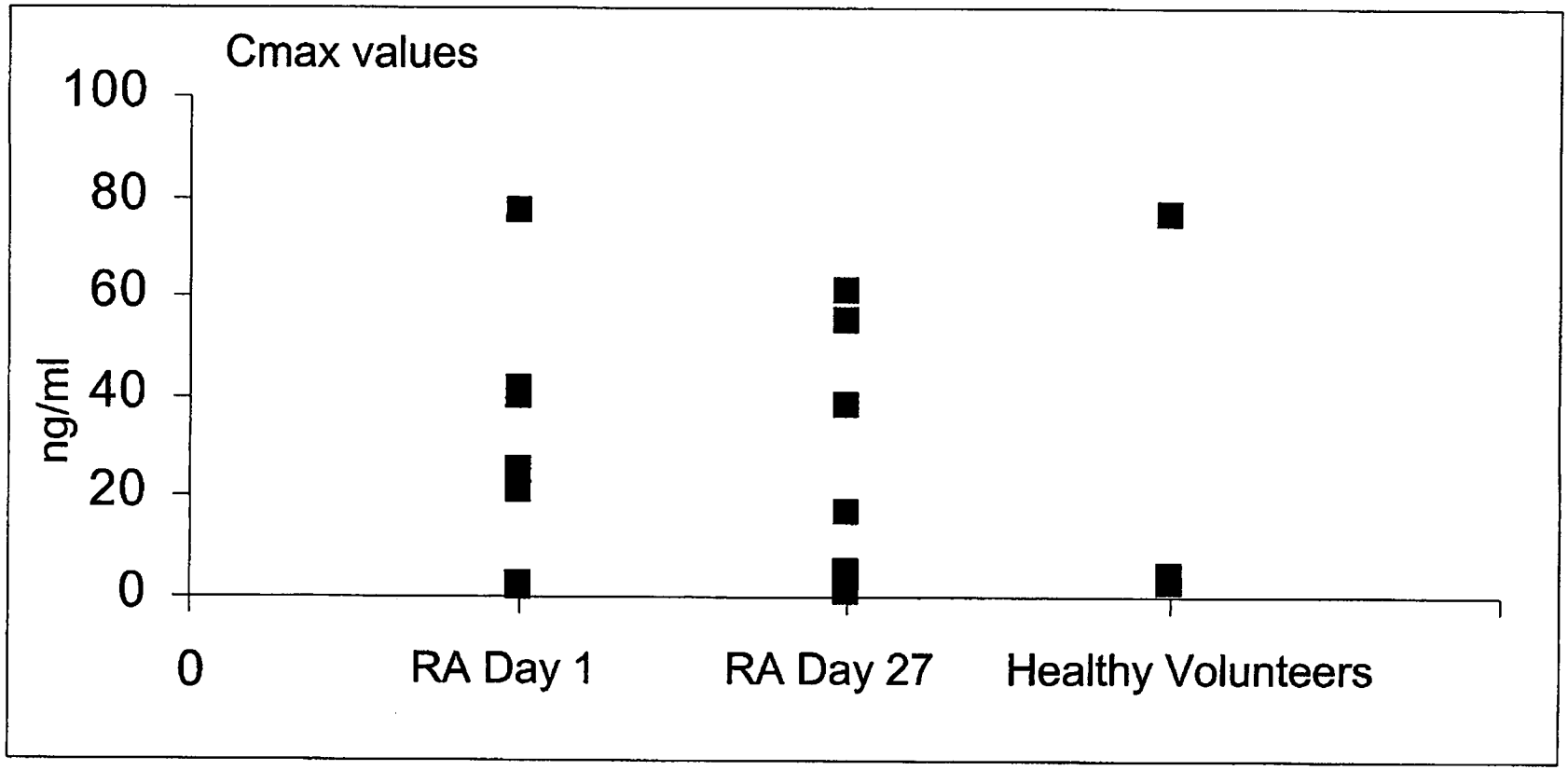
RA Trial Disease Measures

- Patient Assessment
  - Patient Global Assessment of disease (VAS),
    - PMX 53 all patients showed an increase in assessment with 2 showing = 10 points improvement
    - All placebos showed worsening of at least 10 points
  - Patient Assessment of Health (VAS)
    - PMX53 recorded improvement in 3/7 patients
    - Placebo recorded worsening in 3/3 patients
  - VAS pain score
    - PMX53 3/7 (42%) less pain 4/7 unchanged
    - Placebo 1/3 (33%) less pain 2/3 worsened
- Physician Assessment (VAS)
    - PMX53 3/7 (42%) improved and 4/7 unchanged
    - Placebo 1/3 (33%) improved 2/3 worsened

RA Trial Disease Measures

- Patients with elevated baseline CRP
  - 4/4 subjects on PMX53 showed decrease
  - 1/1 subjects on placebo showed increase
- ESR
  - No change observed
- Clinical
  - No trends observed in tender and swollen joints

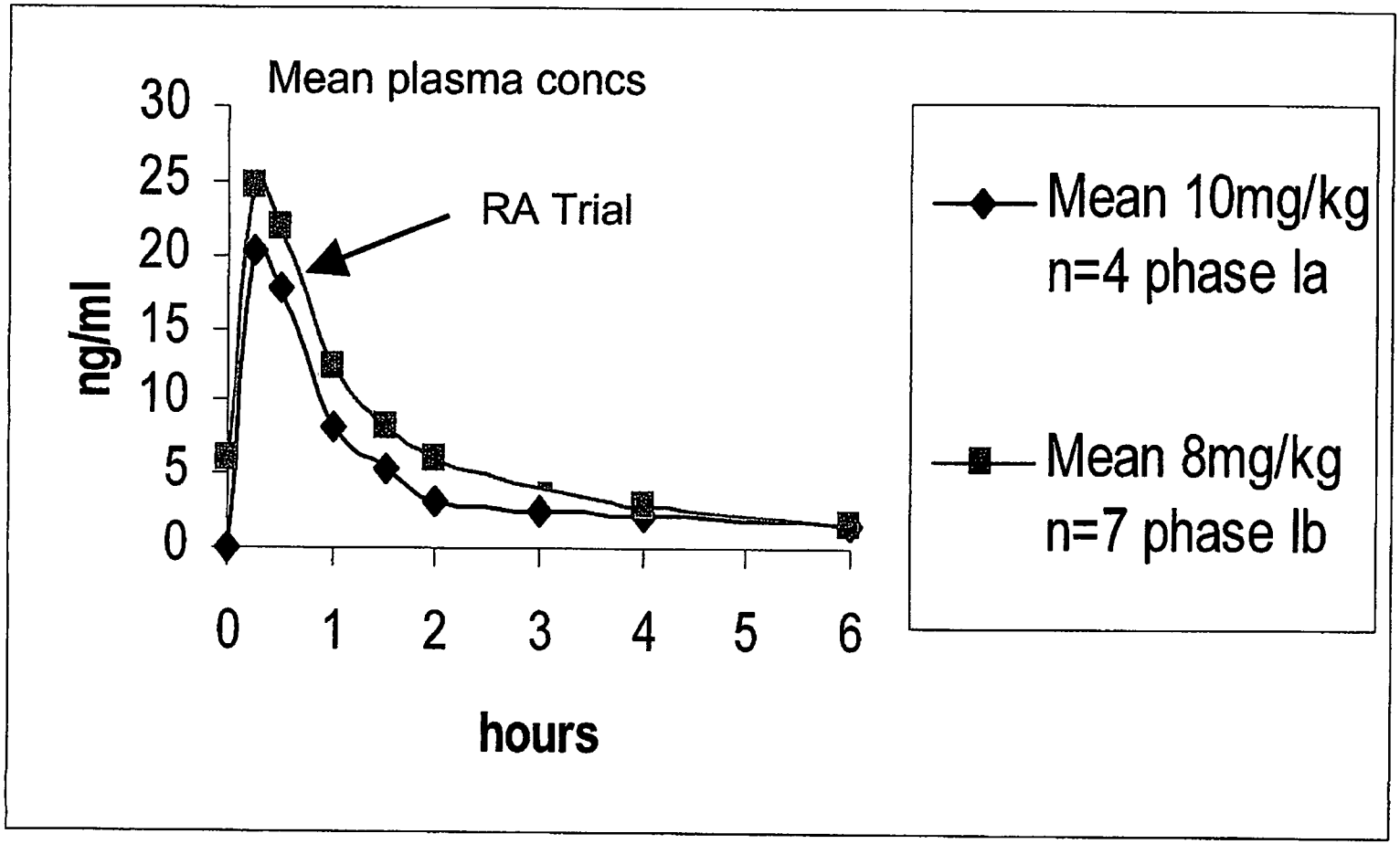
PMX 53 Pharmacokinetics
Range Cmax values



Wide interpatient variability / intrapatient consistent

PMX 53 Pharmacokinetics

PK Profiles similar across studies



PMX 53 Pharmacokinetics

- PK profiles similar to that seen in healthy volunteers
- Blood levels typically higher
- Observe same wide variation between subjects with Cmax ranging from 1 – 40 ng/ml
- No accumulation seen with chronic dosing

PMX53 was Safe and Tolerable

- **PMX53 group (n = 7)**
  - Total No. AEs = 9 across all patients (3 patients no AE's)
  - 2 patients had "moderate" severity AEs - both "unlikely" relationship
  - 4 patients had 7 "mild" severity AEs, 1/7 classed as "possibly" related

- **PLACEBO group (n = 3)**
  - Total No. AEs = 13
  - All mild, 6/13 classed as "possibly" related to drug

* Primary Objective of Study likely to be achieved

RA Trial Protocol

- Randomised, double blind and placebo controlled
- 10 patients with active rheumatoid arthritis on methotrexate for 3 months and a stable dose (5-30 mg/week) for at least one month

  Active disease defined as
  = 6 tender and = 6 swollen joints AND ESR = 28mm/hr
  or CRP = 10mg/L
  or morning stiffness = 45 minutes

- Daily oral dose of 8 mg/kg for 28 days (*recall Phase Ia max single dose 10 mg/kg)*
- Safety evaluation: continuous throughout study
- PK profile Day 1 & Day 27 – trough levels days 7, 14

RA Trial Objectives

Primary objective:

Evaluate the safety and tolerability over 28 days dosing *(recall Phase Ia single dose safety study)*

Secondary objective:

Assess pharmacokinetics of PMX53 in acute and chronic dose setting

Assess biological activity

- synovial biopsy tissue assessment (only at end of study)
- Biochemical markers - CRP, ESR,
- Standard disease measures
- Physician assessment of disease
- Patient self assessment of disease, health and pain

RA Trial Interim Analysis

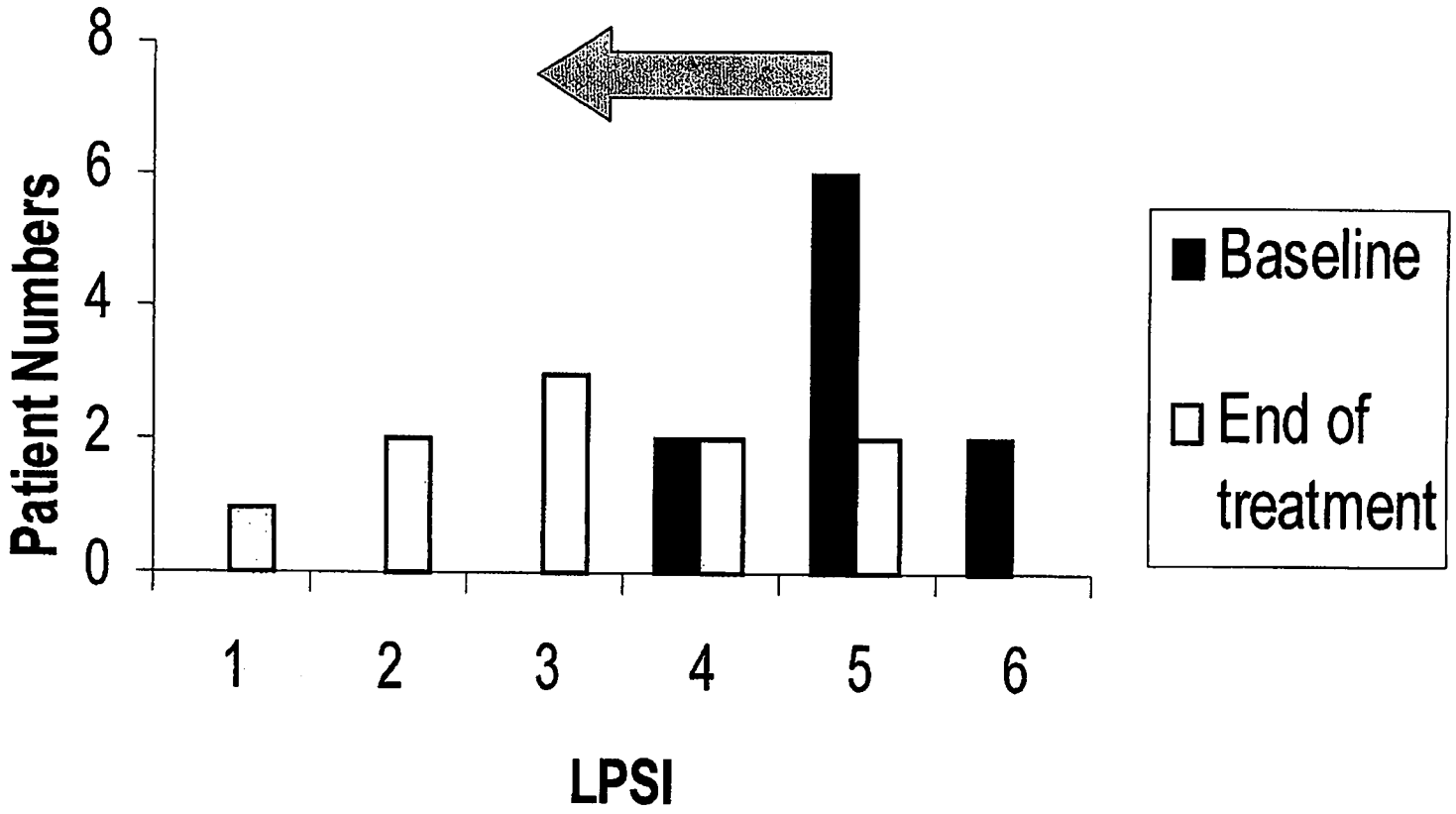
A double-blind study evaluating the safety of PMX53 in comparison to placebo in patients with active rheumatoid arthritis

Psoriasis Trial Summary

- PMX53 Gel (10mg/ml) is safe and tolerable over 56 days
- Data indicates a moderate clinical response

Psoriasis LPSI scores

Lower LPSI scores indicate improvement



Psoriasis Trial

Disease Assessment

- 9/10 patients had improved LPSI score by the end of study

- 1 patient showed improvement of 4 points,
- 4 patients improved by 2 points, and
- 3 patients improved by 1 point

- 8 patients reported improvement in their subjective assessment of the psoriatic lesion

Psoriasis Trial

Safety and Tolerability

- No Serious Adverse Effects
- 16 AE's reported
- Ranging mild to severe (one subject had severe cold and sore throat)
- All classified as not related or unlikely to be related
- Include back pain, headache, sinusitis, head cold, common cold, inflamed psoriasis (1 subject)

Psoriasis Trial

- 10 subjects (for main part of study)
- Mild to moderate chronic plaque type psoriasis, for at least one year
- Target lesion must have
  - Severity Index score (LPSI) of 5-8
  - Area of 10 - 100cm^2;
  - Stable in both extent and severity for two weeks prior to treatment

  Definition LPSI: summed score for
  - erythema, induration and desquamation of target lesion,
  - scale of 0 – 12
  - higher score = more severe disease
  - decrease in LPSI score = improvement

Psoriasis Trial

- Single dose application in healthy volunteers
  - 3 subjects, single application
- Multiple dose application in healthy volunteers
  - 3 subjects, twice daily 4 days
- Multiple dose application in psoriasis patients
  - PMX53 Gel (10mg/ml) will be applied to target lesion twice daily for 56 days

Psoriasis Trial

- Primary Objective
  - Evaluate the safety and tolerability of topically administered PMX53 twice daily for 56 days to target lesions of subjects with psoriasis

- Secondary Objective
  - Evaluate the effect of topical administration of PMX53 on disease status of target lesion

PRM-01-03

Open Label Safety and Tolerability Study of Topical PMX53 in Subjects with Psoriasis

PROMICS DEVELOPMENT REPORT – February 2004

- PRM-01-03 Psoriasis Study Results
- PRM-01-02 RA Interim Data
- Update on Technology Development Plan

March 2004 Development Report

Arthritis Trial

- Recruitment
  - 16 are currently on study
  - 1 subject awaiting screening results
  - 2 further subjects identified as possibility for screening
- Timelines
  - Site committed to complete treatment of all subjects (approximately 20) by end May
  - Synovial tissue processing to be completed within subsequent two months
  - Plan to visit site in the first week of August to discuss interpretation of unblinded data with investigator
  - Full clinical data planned for August board meeting

Formulation of oral dose

- Four companies specialising in solid dose formulation have been approached regarding the development of a formulated oral dose for PMX53
- Proposals have been requested for formulation work that will attempt to:
  - Provide consistent blood levels and minimise subject to subject variation
  - Maximise stomach absorption and bioavailability
- Costing, timelines and recommendations for this contract development to be prepared over next month

Biomarker Development

- Oxidative Burst
  - Promics laboratory have conducted assay validation
  - Data indicates that the assay continues to have considerable assay to assay variation
  - Based on these results it is recommended that the use and investigation of this biomarker in a healthy volunteer study be postponed at least till an improved or alternative formulation of PMX53 is developed
- Alternative LPS model
  - There is precedent for use of this model in healthy volunteer studies to assess activity of new drugs, using clinical and/or cytokine measurements
  - Promics laboratory is investigating viability of this model with PMX53 over the next few months in rats and dogs

Additional development priorities

- Investigate the possibility of alternative manufacturers for PMX53 in China and India with the purpose of addressing Cost of Goods issues

- New formulations for subcutaneous delivery of PMX53 will be tested in Promics laboratories to address injection site toxicity observed in safety and toxicity studies
  - Subcutaneous delivery may present an alternative route of administration and development by a potential purchaser and be appropriate for treatment of some acute inflammatory disorders. This route of administration may also address bioavailability and COGs issues